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(57) Abstract

The present invention provides materials and methods for screening for and treating hereditary lymphedema in human subjects.

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SCREENING AND THERAPY FOR LYMPHATIC DISORDERS INVOLVING THE FLT4 RECEPTOR TYROSINE KINASE (VEGFR-3)

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FIELD OF THE INVENTION

The present invention relates generally to the fields of molecular biology and medicine; more particularly to the areas of genetic screening and the identification and treatment of hereditary disorders; and more particularly to identification and treatment of hereditary lymphedema.

DESCRIPTION OF RELATED ART

The lymphatic system is a complex structure organized in parallel fashion to the circulatory system. In contrast to the circulatory system, which utilizes the heart to pump blood throughout the body, the lymphatic system pumps lymph fluid using the inherent contractility of the lymphatic vessels. The lymphatic vessels are not interconnected in the same manner as the blood vessels, but rather form a set of coordinated structures including the initial lymphatic sinuses [Jeltsch *et al.*, (1997), *Science, 276:*1423-1425; and Castenholz, A., in Olszewski, W.L. (ed.), *Lymph Stasis: Pathophysiology, Diagnosis, and Treatment.* CRC Press: Boca Raton, Florida (1991), pp.15-42] which drain into the lymphatic capillaries and subsequently to the collecting lymphatics which drain into the lymphatic trunks and the thoracic duct which ultimately drains into the venous circulation. The composition of the channels through which lymph passes is varied [Olszewski, W.L., in Olszewski, W.L. (ed), *Lymph Stasis: Pathophysiology, Diagnosis, and Treatment.* CRC Press: Boca Raton, Florida (1991), pp. 235-258; and Kinmonth, J.B., in Kinmonth, J.B. (ed), *The Lymphatics: Diseases, Lymphography and Surgery.* Edward Arnold Publishers: London, England (1972), pp. 82-86], including the single epithelial layers of the initial lymphatics, the

multiple layers of the collecting lymphatics including endothelium, muscular and adventitial layers, and the complex organization of the lymph node. The various organs of the body such as skin, lung, and GI tract have components of the lymphatics with various unique features. [See Ohkuma, M., in Olszewski (1991), *supra*, at pp. 157-190; Uhley, H. and Leeds, S., in Olszewski (1991), *supra*, at pp. 191-210; and Barrowman, J.A., in Olszewski (1991), at pp. 221-234).]

Molecular biology has identified at least a few genes and proteins postulated to have roles mediating the growth and/or embryonic development of the lymphatic system. One such gene/protein is the receptor tyrosine kinase designated Flt4 (fms-like tyrosine kinase 4), cloned from human erythroleukaemia cell and placental cDNA libraries [Aprelikova *et al.*, *Cancer Res., 52:* 746-748 (1992); Galland *et al.*, *Genomics, 13:* 475-478 (1992); Galland *et al.*, *Oncogene, 8:* 1233-1240 (1993); and Pajusola *et al.*, *Cancer Res., 52:*5738-5743 (1992)]. Studies showed that, in mouse embryos, a targeted disruption of the *Flt4* gene leads to a failure of the remodeling of the primary vascular network, and death after embryonic day 9.5 [Dumont *et al.*, *Science, 282:* 946-949 (1998)]. These studies suggested that *Flt4* has an essential role in the development of the embryonic vasculature, before the emergence of the lymphatic vessels. However, additional studies indicated that, during further development, the expression of *Flt4* becomes restricted mainly to lymphatic vessels [Kaipainen, A., *et al.*, *Proc. Natl. Acad. Sci. USA, 92:* 3566-3570 (1995)].

In humans, there are two isoforms of the Flt4 protein, designated as Flt4s (short, Genbank Accession No. X68203) and Flt4l (long, Genbank Accession Nos. X68203 and S66407, SEQ ID NO: 1). The sequence of these isoforms is largely identical, except for divergence that occurs at the carboxyl terminus of the receptor as a result of alternative mRNA splicing at the 3' end. The C-terminus of the long form contains three tyrosyl residues, and one of them (Y1337 (SEQ ID NO: 2)) serves as an autophosphorylation site in the receptor [Fournier *et al.*, *Oncogene, 11*: 921-931 (1995); and Pajusola, *Oncogene, 8:* 2931-2937 (1993)]. Only the long form is detected in human erythroleukaemia (HEL) and in a megakaryoblastic cell line (the DAMI cells), and the mouse *Flt4* gene (Genbank Accession No. L07296) only produces one mRNA transcript, corresponding to Flt4l [Galland *et al.*, *Oncogene, 8*: 1233-1240 (1993); and Pajusola *et al.*, *Cancer Res., 52:* 5738-5743 (1992)]. These findings suggest that the long form of Flt4 may be responsible for most of the

biological properties of this receptor. The Flt4 protein is glycosylated and proteolytically processed in transfected cells [Pajusola *et al.*, *Oncogene, 9:* 3545-3555 (1994)]. During this process, the 175 kD form of the receptor matures to a 195 kD form, which is subsequently cleaved into a 125 kD C-terminal fragment, and a 75 kD extracellular domain-containing fragment, which are linked by disulphide bonding in the mature receptor.

Two growth factors, named vascular endothelial growth factors C and D (VEGF-C and VEGF-D) due to amino acid sequence similarity to earlier-discovered vascular endothelial growth factor, have been shown to bind and activate the tyrosine phosphorylation of Flt4. [Achen *et al.*, *Proc. Natl. Acad. Sci. USA, 95:* 548-553 (1998); and Joukov *et al.*, *EMBO J., 15:* 290-298 (1996)]. Because of Flt4's growth factor binding properties and the fact that Flt4 possesses amino acid sequence similarity to two previously identified VEGF receptors (Flt1/VEGFR-1 and KDR/VEGFR-2), Flt4 has also been designated VEGFR-3, and these terms are used interchangeably herein.

When VEGF-C was intentionally overexpressed under a basal keratin promoter in transgenic mice, a hyperplastic lymphatic vessel network in the skin was observed. [Jeltsch *et al.*, *Science, 276:*1423-1425 (1997).] The results of this study, when combined with the expression pattern of VEGFR-3 in the lymphatic vasculature, suggest that lymphatic growth may be induced by VEGF-C and mediated via VEGFR-3. Notwithstanding the foregoing insights involving one cell surface receptor and the two apparent ligands therefor, little is known about the developmental regulation of the lymphatic system.

Hereditary or primary lymphedema, first described by Milroy in 1892 [Milroy, *N.Y. Med. J., 56*:505-508 (1892)], is a developmental disorder of the lymphatic system which leads to a disabling and disfiguring swelling of the extremities. Hereditary lymphedema generally shows an autosomal dominant pattern of inheritance with reduced penetrance, variable expression, and variable age-at-onset [Greenlee *et al.*, *Lymphology, 26*:156-168 (1993)]. Swelling may appear in one or all limbs, varying in degree and distribution. If untreated, such swelling worsens over time. In rare instances, angiosarcoma may develop in affected tissues [Offori *et al.*, *Clin. Exp. Dermatol., 18*:174-177 (1993)]. Despite having been described over a century ago, little progress has been made in understanding the mechanisms causing lymphedema.

A long-felt need exists for the identification of the presumed genetic variations that underlie hereditary lymphedema, to permit better informed genetic counseling in affected families, earlier diagnosis and treatment, and the development of more targeted and effective lymphedema therapeutic regimens. In addition, identification of genetic markers and high risk members of lymphedema families facilitates the identification and management of environmental factors that influence the expression and severity of a lymphedema phenotype.

SUMMARY OF THE INVENTION

The present invention provides materials and methods that address one or more of the long-felt needs identified above by identifying a genetic marker that correlates and is posited to have a causative role in the development of hereditary lymphedema. The invention is based in part on the discovery that, in several families with members afflicted with hereditary lymphedema, the lymphedema phenotype correlates with genetic markers localized to chromosome 5q34-q35; and that in at least one such family, a missense mutation in theVEGFR-3 gene (which maps to chromosome 5q34-q35) exists that appears to behave in a dominant negative manner to interrupt tyrosine kinase signaling of the receptor. In view of the fact that VEGFR-3 acts as a high affinity receptor for vascular endothelial growth factor C (VEGF-C), a growth factor whose effects include modulation of the growth of the lymphatic vascular network, these linkage and biochemical studies provide an important marker for determining a genetic predisposition for lymphedema in healthy individuals; and for diagnosing hereditary lymphedema in symptomatic individuals. Materials and methods for performing such genetic analyses are considered aspects of the present invention.

Thus, the invention provides genetic screening procedures that entail analyzing a person's genome -- in particular their *VEGFR-3* alleles -- to determine whether the individual possesses a genetic characteristic found in other individuals that are considered to be afflicted with, or at risk for, developing hereditary lymphedema.

For example, in one embodiment, the invention provides a method for determining a hereditary lymphedema development potential in a human subject comprising the steps of analyzing the coding sequence of the VEGFR-3 genes from the human subject; and determining hereditary lymphedema development potential in said human subject from the analyzing step.

In another embodiment, the invention provides a method of screening a human subject for an increased risk of developing a lymphatic disorder, comprising the steps of: (a) assaying nucleic acid of a human subject to determine a presence or an absence of a mutation altering the encoded VEGFR-3 amino acid sequence or expression of at least one VEGFR-3 allele; and (b) screening for an increased risk of developing a lymphatic disorder from the presence or absence of said mutation.

By "human subject" is meant any human being, human embryo, or human fetus. It will be apparent that methods of the present invention will be of particular interest to individuals that have themselves been diagnosed with lymphedema or have relatives that have been diagnosed with lymphedema.

By "screening for an increased risk" is meant determination of whether a genetic variation exists in the human subject that correlates with a greater likelihood of developing lymphedema than exists for the human population as a whole, or for a relevant racial or ethnic human sub-population to which the individual belongs. Both positive and negative determinations (i.e., determinations that a genetic predisposition marker is present or is absent) are intended to fall within the scope of screening methods of the invention. In preferred embodiments, the presence of a mutation altering the sequence or expression of at least one *Flt4* receptor tyrosine kinase allele in the nucleic acid is correlated with an increased risk of developing a lymphatic disorder, whereas the absence of such a mutation is reported as a negative determination.

By "lymphatic disorder" is meant any clinical condition affecting the lymphatic system, including but not limited to lymphedemas, lymphangiomas, lymphangiosarcomas, lymphangiomatosis, lymphangiectasis, and cystic hygroma. Preferred embodiments are methods of screening a human subject for an increased risk of developing a lymphedema disorder, *i.e.*, any disorder that physicians would diagnose as lymphedema and that is characterized by swelling associated with lymph accumulation, other than lymphedemas for which non-genetic causes (e.g., parasites, surgery) are known. By way of example, lymphedema disorders include Milroy-Nonne (OMIM 153100) syndrome-early onset lymphedema [Milroy, *N.Y. Med. J.*, *56*:505-508 (1892); and Dale, *J. Med. Genet.*, *22*: 274-278 (1985)] and lymphedema praecox (Meige syndrome, OMIM 153200)-late onset lymphedema [Lewis *et al.*, *J. Ped.*, *104*:641-648 (1984); Holmes *et al.*, *Pediatrics 61*:575-579 (1978); and Wheeler *et al.*,

*Plastic Reconstructive Surg., 67:*362-364 (1981)] which generally are described as separate entities, both characterized by dominant inheritance. However, there is confusion in the literature about the separation of these disorders. In Milroy's syndrome, the presence of edema, which is usually more severe in the lower extremities, is seen from birth. Lymphedema praecox presents in a similar fashion but the onset of swelling is usually around puberty. Some cases have been reported to develop in the post-pubertal period. In the particular analyses described herein, the lymphedema families showing linkage to 5q34-q35 show an early onset for most affected individuals, but individuals in these pedigrees have presented during or after puberty.

The "assaying" step of the invention may involve any techniques available for analyzing nucleic acid to determine its characteristics, including but not limited to well-known techniques such as single-strand conformation polymorphism analysis (SSCP) [Orita *et al., Proc Natl. Acad. Sci. USA, 86*: 2766-2770 (1989)]; heteroduplex analysis [White *et al., Genomics, 12*: 301-306 (1992)]; denaturing gradient gel electrophoresis analysis [Fischer *et al., Proc. Natl. Acad. Sci. USA, 80*: 1579-1583 (1983); and Riesner *et al., Electrophoresis, 10*: 377-389 (1989)]; DNA sequencing; RNase cleavage [Myers *et al., Science, 230*: 1242-1246 (1985)]; chemical cleavage of mismatch techniques [Rowley *et al., Genomics, 30*: 574-582 (1995); and Roberts *et al., Nucl. Acids Res., 25*: 3377-3378 (1997)]; restriction fragment length polymorphism analysis; single nucleotide primer extension analysis [Shumaker *et al., Hum. Mutat., 7*: 346-354 (1996); and Pastinen *et al., Genome Res., 7*: 606-614 (1997)]; 5' nuclease assays [Pease *et al., Proc. Natl. Acad. Sci. USA, 91*:5022-5026 (1994)]; DNA Microchip analysis [Ramsay, G., *Nature Biotechnology, 16*: 40-48 (1999); and Chee *et al.*, U.S. Patent No. 5,837,832]; and ligase chain reaction [Whiteley *et al.*, U.S. Patent No. 5,521,065]. [See generally, Schafer and Hawkins, *Nature Biotechnology, 16*: 33-39 (1998).] All of the foregoing documents are hereby incorporated by reference in their entirety.

In one preferred embodiment, the assaying involves sequencing of nucleic acid to determine nucleotide sequence thereof, using any available sequencing technique. [See, *e.g.*, Sanger *et al., Proc. Natl. Acad. Sci. (USA), 74*: 5463-5467 (1977) (dideoxy chain termination method); Mirzabekov, *TIBTECH, 12*: 27-32 (1994) (sequencing by hybridization); Drmanac *et al., Nature Biotechnology, 16*: 54-58

(1998); U.S. Patent No. 5,202,231; and *Science, 260*: 1649-1652 (1993) (sequencing by hybridization); Kieleczawa *et al., Science, 258*: 1787-1791 (1992) (sequencing by primer walking); (Douglas *et al., Biotechniques, 14*: 824-828 (1993) (Direct sequencing of PCR products); and Akane *et al., Biotechniques 16*: 238-241 (1994); Maxam and Gilbert, *Meth. Enzymol., 65*: 499-560 (1977) (chemical termination sequencing), all incorporated herein by reference.] The analysis may entail sequencing of the entire *VEGFR-3* gene genomic DNA sequence, or portions thereof; or sequencing of the entire *VEGFR-3* coding sequence or portions thereof. In some circumstances, the analysis may involve a determination of whether an individual possesses a particular *VEGFR-3* allelic variant, in which case sequencing of only a small portion of nucleic acid -- enough to determine the sequence of a particular codon characterizing the allelic variant -- is sufficient. This approach is appropriate, for example, when assaying to determine whether one family member inherited the same allelic variant that has been previously characterized for another family member, or, more generally, whether a person's genome contains an allelic variant that has been previously characterized and correlated with heritable lymphedema.

In another embodiment, the assaying step comprises performing a hybridization assay to determine whether nucleic acid from the human subject has a nucleotide sequence identical to or different from one or more reference sequences. In a preferred embodiment, the hybridization involves a determination of whether nucleic acid derived from the human subject will hybridize with one or more oligonucleotides, wherein the oligonucleotides have nucleotide sequences that correspond identically to a portion of the VEGFR-3 gene sequence, preferably the VEGFR-3 coding sequence set forth in SEQ ID NO: 1, or that correspond identically except for one mismatch. The hybridization conditions are selected to differentiate between perfect sequence complementarity and imperfect matches differing by one or more bases. Such hybridization experiments thereby can provide single nucleotide polymorphism sequence information about the nucleic acid from the human subject, by virtue of knowing the sequences of the oligonucleotides used in the experiments.

Several of the techniques outlined above involve an analysis wherein one performs a polynucleotide migration assay, *e.g.*, on a polyacrylamide electrophoresis gel, under denaturing or non-denaturing conditions. Nucleic acid derived from the human subject is subjected to gel electrophoresis, usually adjacent to

one or more reference nucleic acids, such as reference VEGFR-3 sequences having a coding sequence identical to all or a portion of SEQ ID NO: 1, or identical except for one known polymorphism. The nucleic acid from the human subject and the reference sequence(s) are subjected to similar chemical or enzymatic treatments and then electrophoresed under conditions whereby the polynucleotides will show a differential migration pattern, unless they contain identical sequences. [See generally Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, New York: John Wiley & Sons, Inc. (1987-1999); and Sambrook *et al.*, (eds.), Molecular Cloning, *A Laboratory Manual*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press (1989), both incorporated herein by reference in their entirety.]

In the context of assaying, the term "nucleic acid of a human subject" is intended to include nucleic acid obtained directly from the human subject (*e.g.*, DNA or RNA obtained from a biological sample such as a blood, tissue, or other cell or fluid sample); and also nucleic acid derived from nucleic acid obtained directly from the human subject. By way of non-limiting examples, well known procedures exist for creating cDNA that is complementary to RNA derived from a biological sample from a human subject, and for amplifying (*e.g.*, via polymerase chain reaction (PCR)) DNA or RNA derived from a biological sample obtained from a human subject. Any such derived polynucleotide which retains relevant nucleotide sequence information of the human subject's own DNA/RNA is intended to fall within the definition of "nucleic acid of a human subject" for the purposes of the present invention.

In the context of assaying, the term "mutation" includes addition, deletion, and/or substitution of one or more nucleotides in the *VEGFR-3* gene sequence. The invention is demonstrated by way of non-limiting examples set forth below that identify several mutations in *VEGFR-3*, including single nucleotide polymorphisms that introduce missense mutations into the *VEGFR-3* coding sequence (as compared to the *VEGFR-3* cDNA sequence set forth in SEQ ID NO: 1) and other polymorphisms that occur in introns and that are identifiable via sequencing, restriction fragment length polymorphism, or other techniques. Example 2 provides an assay to determine whether a *VEGFR-3* mutation inhibits VEGFR-3 signaling. Additional assays to study both ligand binding and signaling activities of VEGFR-3 are disclosed, *e.g.*, in U.S. Patent No. 5,776,755 and International Patent Publication No. WO 98/33917, published 06 August 1998, both of which are incorporated herein by

reference in their entirety. Evidence that a VEGFR-3 mutation inhibits VEGFR-3 signaling is evidence that the mutation may have a causative role in lymphedema phenotype. However, even mutations that have no apparent causative role may serve as useful markers for heritable lymphedema, provided that the appearance of the mutation correlates reliably with the appearance of lymphedema.

In a related embodiment, the invention provides a method of screening for a VEGFR-3 hereditary lymphedema genotype in a human subject, comprising the steps of: (a) providing a biological sample comprising nucleic acid from a human subject; (b) analyzing the nucleic acid for the presence of a mutation or mutations in a VEGFR-3 allele in the nucleic acid of the human subject; (c) determining a VEGFR-3 genotype from said analyzing step; and (d) correlating the presence of a mutation in a VEGFR-3 allele with a hereditary lymphedema genotype. In a preferred embodiment, the biological sample is a cell sample containing human cells that contain genomic DNA of the human subject.

Although more time consuming and expensive than methods involving nucleic acid analysis, the invention also may be practiced by assaying protein of a human subject to determine the presence or absence of an amino acid sequence variation in VEGFR-3 protein from the human subject. Such protein analyses may be performed, e.g., by fragmenting VEGFR-3 protein via chemical or enzymatic methods and sequencing the resultant peptides; or by Western analyses using an antibody having specificity for a particular allelic variant of VEGFR-3.

The invention also provides materials that are useful for performing methods of the invention. For example, the present invention provides oligonucleotides useful as probes in the many analyzing techniques described above. In general, such oligonucleotide probes comprise 6, 7, 8, 9, 10 , 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides that have a sequence that is identical, or exactly complementary, to a portion of a human VEGFR-3 gene sequence, or that is identical or exactly complementary except for one nucleotide substitution. In a preferred embodiment, the oligonucleotides have a sequence that corresponds in the foregoing manner to a human VEGFR-3 coding sequence, and in particular, the VEGFR-3 coding sequence set forth in SEQ ID NO: 1. In one variation, an oligonucleotide probe of the invention is purified and isolated. In another

variation, the oligonucleotide probe is labeled, *e.g.*, with a radioisotope, chromophore, or fluorophore. In yet another variation, the probe is covalently attached to a solid support. [See generally Ausubel *et al.* And Sambrook *et al.*, *supra.*]

In a particularly preferred embodiment, the invention comprises an oligonucleotide probe useful for detecting the P1114L mutation (missense mutation at nucleotide 3360 of SEQ ID NO: 1, causing a proline to leucine change at residue 1114 in SEQ ID NO: 2) that has been characterized herein in affected individuals of one lymphedema family. For example, the invention provides oligonucleotides comprising anywhere from 6 to 50 nucleotides that have a sequence that is identical to, or exactly complementary to, a portion of the human VEGFR-3 coding sequence set forth in SEQ ID NO: 1, except for a nucleotide substitution corresponding to nucleotide 3360 of SEQ ID NO: 1. Such oligonucleotides may be generically described by the formula X_nYZ_m or its complement; where n and m are integers from 0 to 49; where $5 \leq (n + m) \leq 49$; where X_n is a stretch of n nucleotides identical to a first portion of SEQ ID NO: 1 and Z_m is a stretch of m nucleotides identical to a second portion of SEQ ID NO: 1, wherein the first and second portions are separated in SEQ ID NO: 1 by one nucleotide; and wherein Y represents a nucleotide other than the nucleotide that separates the first and second portions of SEQ ID NO: 1. For example, where X_n represents 0 to 49 nucleotides immediately upstream (5') of nucleotide 3360 of SEQ ID NO: 1 and Z_m represents 0 to 49 nucleotides immediately downstream (3') of nucleotide 3360 of SEQ ID NO: 1, Y represents a nucleotide other than cytosine, since a cytosine nucleotide is found at position 3360 of SEQ ID NO: 1. In a preferred embodiment, Y is a thymine nucleotide.

In a related embodiment, the invention provides a kit comprising at least two such oligonucleotide probes. Preferably, the two or more probes are provided in separate containers, or attached to separate solid supports, or attached separately to the same solid support, e.g., on a DNA microchip.

In still another related embodiment, the invention provides an array of oligonucleotide probes immobilized on a solid support, the array having at least 4 probes, preferably at least 100 probes, and preferably up to 100,000, 10,000, or 1000 probes, wherein each probe occupies a separate known site in the array. In a preferred embodiment, the array includes probe sets comprising two to four probes, wherein one probe is exactly identical or exactly complementary to a human VEGFR-3 coding

sequence, and the other one to three members of the set are exactly identical to the first member , but for at least one different nucleotide, which different nucleotide is located in the same position in each of the one to three additional set members. In one preferred embodiment, the array comprises several such sets of probes, wherein the sets correspond to different segments of the human VEGFR-3 gene sequence. In a highly preferred embodiment, the array comprises enough sets of oligonucleotides of length N to correspond to every particular N-mer sequence of the VEGFR-3 gene, where N is preferably 6 to 25 and more preferably 9 to 20. Materials and methods for making such probes are known in the art and are described, for example, in U.S. Patent Nos. 5,837,832, 5,202,231, 5,002,867, and 5,143,854.

Moreover, the discoveries which underlie the present invention identify a target for therapeutic intervention in cases of hereditary lymphedema. The causative mutation in the family that has been studied in greatest detail is a mutation that appears to result in VEGFR-3 signaling that is reduced in heterozygous affected individuals, but not completely eliminated. This data supports a therapeutic indication for administration of agents, such as VEGFR-3 ligand polypeptides, that will induce VEGFR-3 signaling in the lymphatic endothelia of affected individuals to effect improvement in the structure and function of the lymphatic vasculature of such individuals. In addition, therapeutic gene therapy, to replace defective VEGFR-3 alleles or increase production of VEGFR-3 ligand polypeptides *in vivo*, is envisioned as an aspect of the invention.

Thus, in yet another aspect, the invention provides a therapeutic or prophylactic method of treatment for lymphedema, comprising the step of administering to a mammalian subject in need of therapeutic or prophylactic treatment for lymphedema a composition comprising a compound effective to induce intracellular signaling of VEGFR-3 in lymphatic endothelial cells that express said receptor. In a preferred embodiment, the compound comprises a polypeptide ligand for VEGFR-3, or a polynucleotide encoding such a ligand, wherein the polynucleotide is administered in a form that results in transcription and translation of the polynucleotide in the mammalian subject to produce the ligand *in vivo*. In another preferred embodiment, the compound comprises any small molecule that is capable of binding to the VEGFR-3 receptor extracellular domain and inducing intracellular signaling.

For example, the invention provides a therapeutic or prophylactic method of treatment for lymphedema, comprising the step of administering to a mammalian subject in need of therapeutic or prophylactic treatment for lymphedema a composition comprising a polynucleotide, the polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor C (VEGF-C) polypeptide. In a preferred embodiment, the subject is a human subject.

While it is contemplated that the VEGF-C polynucleotide could be administered purely as a prophylactic treatment to prevent lymphedema in subjects at risk for developing lymphedema, it is contemplated in a preferred embodiment that the polynucleotide be administered to subjects afflicted with lymphedema, for the purpose of ameliorating its symptoms (e.g., swelling due to the accumulation of lymph). The polynucleotide is included in the composition in an amount and in a form effective to promote expression of a VEGF-C polypeptide in or near the lymphatic endothelia of the mammalian subject, to stimulate VEGFR-3 signaling in the lymphatic endothelia of the subject.

In a preferred embodiment, the mammalian subject is a human subject. Practice of methods of the invention in other mammalian subjects, especially mammals that are conventionally used as models for demonstrating therapeutic efficacy in humans (e.g., primate, porcine, canine, equine, murine, or rabbit animals), also is contemplated. Several potential animal models for hereditary lymphedema have been described in the literature. [See, e.g., Lyon *et al., Mouse News Lett. 71*: 26 (1984), *Mouse News Lett. 74*: 96 (1986), and *Genetic variants and strains of the laboratory mouse*, 2nd ed., New York: Oxford University Press (1989), p. 70 (*Chylous ascites* mouse); Dumont *et al., Science, 282*: 946-949 (1998) (heterozygous VEGFR-3 knockout mouse); Patterson *et al.*, "Hereditary Lymphedema," *Comparative Pathology Bulletin, 3*: 2 (1971) (canine hereditary lymphedema model); van der Putte, "Congenital Hereditary Lymphedema in the Pig," *Lympho, 11*: 1-9 (1978); and Campbell-Beggs *et al.*, "Chyloabdomen in a neonatal foal," *Veterinary Record, 137*: 96-98 (1995).] Those models which are determined to have analogous mutations to the VEGFR-3 gene are preferred. In another embodiment, "knock in" homologous recombination genetic engineering strategies are used to create an animal model (e.g., a mouse model) having a VEGFR-3 allelic variation analogous to the human variations described herein. [See, *e.g.*, Partanen *et al., Genes & Development, 12*: 2332-2344

(1998) (gene targeting to introduce mutations into a receptor protein (FGFR-1) in mice).] Such mice can also be bread to the heterozygous VEGFR-3 knockout mice or *Chy* mice described above to further modify the phenotypic severity of the lymphedema disease.

For the practice of methods of the invention, the term "VEGF-C polypeptide" is intended to include any polypeptide that has a VEGF-C or VEGF-C analog amino acid sequence (as defined elsewhere herein in greater detail) and that is able to bind the VEGFR-3 extracellular domain and stimulate VEGFR-3 signaling *in vivo*. The term "VEGF-C polynucleotide" is intended to include any polynucleotide (*e.g.*, DNA or RNA, single- or double-stranded) comprising a nucleotide sequence that encodes a VEGF-C polypeptide. Due to the well-known degeneracy of the genetic code, multiple VEGF-C polynucleotide sequences exist that encode any selected VEGF-C polypeptide.

For treatment of humans, VEGF-C polypeptides with an amino acid sequence of a human VEGF-C are highly preferred, and polynucleotides comprising a nucleotide sequence of a human VEGF-C cDNA are highly preferred. By "human VEGF-C" is meant a polypeptide corresponding to a naturally occurring protein (prepro-protein, partially-processed protein, or fully-processed mature protein) encoded by any allele of the human VEGF-C gene, or a polypeptide comprising a biologically active fragment of a naturally-occurring mature protein. By way of example, a human VEGF-C comprises a continuous portion of the amino acid sequence set forth in SEQ ID NO: 4 sufficient to permit the polypeptide to bind and stimulate VEGFR-3 phosphorylation in cells that express such receptors. A polypeptide comprising amino acids 131-211 of SEQ ID NO: 4 is specifically contemplated. For example, polypeptides having an amino acid sequence comprising a continuous portion of SEQ ID NO: 4, the continuous portion having, as its amino terminus, an amino acid selected from the group consisting of positions 30-131 of SEQ ID NO: 4, and having, as its carboxyl terminus, an amino acid selected from the group consisting of positions 211-419 of SEQ ID NO: 4 are contemplated. An amino terminus selected from the group consisting of positions 102-131 of SEQ ID NO: 4 is preferred, and an amino terminus selected from the group consisting of positions 103-113 of SEQ ID NO: 4 is highly preferred. Likewise, a carboxyl terminus selected from the group consisting of positions 211-227 of SEQ ID NO: 4 is preferred. As

stated above, the term "human VEGF-C" also is intended to encompass polypeptides encoded by allelic variants of the human VEGF-C characterized by the sequences set forth in SEQ ID NOs: 3 & 4.

Moreover, since the therapeutic VEGF-C is to be administered as recombinant VEGF-C or indirectly via somatic gene therapy, it is within the skill in the art to make and use analogs of human VEGF-C (and polynucleotides that encode such analogs) wherein one or more amino acids have been added, deleted, or replaced with other amino acids, especially with conservative replacements, and wherein the VEGFR-3-stimulatory biological activity has been retained. Analogs that retain VEGFR-3-stimulatory VEGF-C biological activity are contemplated as VEGF-C polypeptides for use in the present invention. In a preferred embodiment, analogs having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 such modifications and that retain VEGFR-3-stimulatory VEGF-C biological activity are contemplated as VEGF-C polypeptides for use in the present invention. Polynucleotides encoding such analogs are generated using conventional PCR, site-directed mutagenesis, and chemical synthesis techniques.

Also contemplated as VEGF-C polypeptides are non-human mammalian or avian VEGF-C polypeptides and polynucleotides. By "mammalian VEGF-C" is meant a polypeptide corresponding to a naturally occurring protein (prepro-protein, partially-processed protein, or fully-processed mature protein) encoded by any allele of a VEGF-C gene of any mammal, or a polypeptide comprising a biologically active fragment of a mature protein. The term "mammalian VEGF-C polypeptide" is intended to include analogs of mammalian VEGF-C's that possess the *in vivo* VEGFR-3-stimulatory effects of the mammalian VEGF-C.

Irrespective of which encoded VEGF-C polypeptide is chosen, any VEGF-C polynucleotide gene therapy pharmaceutical encoding it preferably comprises a nucleotide sequence encoding a secretory signal peptide fused in-frame with the VEGF-C polypeptide sequence. The secretory signal peptide directs secretion of the VEGF-C polypeptide by the cells that express the polynucleotide, and is cleaved by the cell from the secreted VEGF-C polypeptide. For example, the VEGF-C polynucleotide could encode the complete prepro-VEGF-C sequence set forth in SEQ ID NO: 4; or could encode the VEGF-C signal peptide fused in-frame to a sequence encoding a fully-processed VEGF-C (*e.g.*, amino acids 103-227 of SEQ ID NO: 4) or

VEGF-C analog. Moreover, there is no requirement that the signal peptide be derived from VEGF-C. The signal peptide sequence can be that of another secreted protein, or can be a completely synthetic signal sequence effective to direct secretion in cells of the mammalian subject.

In one embodiment, the VEGF-C polynucleotide of the invention comprises a nucleotide sequence that will hybridize to a polynucleotide that is complementary to the human VEGF-C cDNA sequence specified in SEQ ID NO: 3 under the following exemplary stringent hybridization conditions: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM $Na•PO_4$, pH 6.8; and washing in 1X SSC at 55°C for 30 minutes; and wherein the nucleotide sequence encodes a polypeptide that binds and stimulates human VEGFR-3. It is understood that variation in these exemplary conditions occur based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. [*See* Sambrook *et al., Molecular Cloning: A Laboratory Manual* (Second ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989) §§ 9.47-9.51.]

In preferred embodiments, the VEGF-C polynucleotide further comprises additional sequences to facilitate the VEGF-C gene therapy. In one embodiment, a "naked" VEGF-C transgene (i.e., a transgene without a viral, liposomal, or other vector to facilitate transfection) is employed for gene therapy. In this embodiment, the VEGF-C polynucleotide preferably comprises a suitable promoter and/or enhancer sequence (*e.g.*, cytomegalovirus promoter/enhancer [Lehner *et al., J. Clin. Microbiol., 29*:2494-2502 (1991); Boshart *et al., Cell, 41*:521-530 (1985)]; Rous sarcoma virus promoter [Davis *et al., Hum. Gene Ther.*, 4:151 (1993)]; Tie promoter [Korhonen *et al., Blood, 86(5)*: 1828-1835 (1995)]; or simian virus 40 promoter) for expression in the target mammalian cells, the promoter being operatively linked upstream (i.e., 5') of the VEGF-C coding sequence. The VEGF-C polynucleotide also preferably further includes a suitable polyadenylation sequence (*e.g.*, the SV40 or human growth hormone gene polyadenylation sequence) operably linked downstream (i.e., 3') of the VEGF-C coding sequence. The polynucleotide may further optionally comprise sequences whose only intended function is to facilitate large-scale production of the vector, *e.g.*, in bacteria, such as a bacterial origin of replication and a sequence encoding a selectable marker. However, in a preferred

embodiment, such extraneous sequences are at least partially cleaved off prior to administration to humans according to methods of the invention. One can manufacture and administer such polynucleotides to achieve successful gene therapy using procedures that have been described in the literature for other transgenes. See, *e.g.*, Isner *et al., Circulation, 91*: 2687-2692 (1995); and Isner *et al., Human Gene Therapy, 7*: 989-1011 (1996); incorporated herein by reference in the entirety.

Any suitable vector may be used to introduce the VEGF-C transgene into the host. Exemplary vectors that have been described in the literature include replication-deficient retroviral vectors, including but not limited to lentivirus vectors [Kim *et al., J. Virol., 72(1)*: 811-816 (1998); Kingsman & Johnson, *Scrip Magazine*, October, 1998, pp. 43-46.]; adeno-associated viral vectors [Gnatenko *et al., J. Investig. Med., 45*: 87-98 (1997)]; adenoviral vectors [See, *e.g.*, U.S. Patent No. 5,792,453; Quantin *et al., Proc. Natl. Acad. Sci. USA, 89*: 2581-2584 (1992); Stratford-Perricadet *et al., J. Clin. Invest., 90*: 626-630 (1992); and Rosenfeld *et al., Cell, 68*: 143-155 (1992)]; Lipofectin-mediated gene transfer (BRL); liposomal vectors [See, *e.g.*, U.S. Patent No. 5,631,237 (Liposomes comprising Sendai virus proteins)] ; and combinations thereof. All of the foregoing documents are incorporated herein by reference in the entirety. Replication-deficient adenoviral vectors constitute a preferred embodiment.

In embodiments employing a viral vector, preferred polynucleotides still include a suitable promoter and polyadenylation sequence as described above. Moreover, it will be readily apparent that, in these embodiments, the polynucleotide further includes vector polynucleotide sequences (*e.g.*, adenoviral polynucleotide sequences) operably connected to the sequence encoding a VEGF-C polypeptide.

Thus, in one embodiment the composition to be administered comprises a vector, wherein the vector comprises the VEGF-C polynucleotide. In a preferred embodiment, the vector is an adenovirus vector. In a highly preferred embodiment, the adenovirus vector is replication-deficient, i.e., it cannot replicate in the mammalian subject due to deletion of essential viral-replication sequences from the adenoviral genome. For example, the inventors contemplate a method wherein the vector comprises a replication-deficient adenovirus, the adenovirus comprising the VEGF-C polynucleotide operably connected to a promoter and flanked on either end by adenoviral polynucleotide sequences.

The composition to be administered according to methods of the invention preferably comprises (in addition to the polynucleotide or vector) a pharmaceutically-acceptable carrier solution such as water, saline, phosphate-buffered saline, glucose, or other carriers conventionally used to deliver therapeutics intravascularly. Multi-gene therapy is also contemplated, in which case the composition optionally comprises both the VEGF-C polynucleotide/vector and another polynucleotide/vector. As described in greater detail below, a VEGF-D transgene is a preferred candidate for co-administration with the VEGF-C transgene.

The "administering" that is performed according to the present method may be performed using any medically-accepted means for introducing a therapeutic directly or indirectly into a mammalian subject to reach the lymph or the lymphatic system, including but not limited to injections; oral ingestion; intranasal or topical administration; and the like. In a preferred embodiment, administration of the composition comprising the VEGF-C polynucleotide is performed intravascularly, such as by intravenous or intra-arterial injection, or by subcutaneous injection or local depot administration. In a highly preferred embodiment, the composition is administered locally, *e.g.*, to the site of swelling.

In still another variation, endothelial cells or endothelial progenitor cells are transfected *ex vivo* with a wild type VEGFR-3 transgene, and the transfected cells are administered to the mammalian subject.

In another aspect, the invention provides a therapeutic or prophylactic method of treating for lymphedema, comprising the step of administering to a mammalian subject in need of treatment for lymphedema a composition comprising a VEGF-C polypeptide, in an amount effective to treat or prevent swelling associated with lymphedema. Administration via one or more intravenous or subcutaneous injections is contemplated. Co-administration of VEGF-C polynucleotides and VEGF-C polypeptides is also contemplated.

In yet another embodiment, the invention provides the use of a VEGF-C polynucleotide or VEGF-C polypeptide for the manufacture of a medicament for the treatment or prevention of lymphedema.

In still another embodiment, the invention provides a therapeutic or prophylactic method of treatment for lymphedema, comprising the step of administering to a mammalian subject in need of therapeutic or prophylactic treatment

of lymphedema a composition comprising a polynucleotide, the polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor D (VEGF-D) polypeptide. Such methods are practiced essentially as described herein with respect to VEGF-C-encoding polynucleotides, except that polynucleotides encoding VEGF-D are employed. A detailed description of the human VEGF-D gene and protein are provided in Achen, *et al., Proc. Nat'l Acad. Sci. U.S.A., 95(2)*: 548-553 (1998); International Patent Publication No. WO 98/07832, published 26 February 1998; and in Genbank Accession No. AJ000185, all incorporated herein by reference. A cDNA and deduced amino acid sequence for prepro-VEGF-D is set forth herein in SEQ ID NOs: 5 and 6. Of course, due to the well-known degeneracy of the genetic code, multiple VEGF-D encoding polynucleotide sequence exist, any of which may be employed according to the methods taught herein.

As described herein in detail with respect to VEGF-C, the use of polynucleotides that encode VEGF-D fragments, VEGF-D analogs, VEGF-D allelic and interspecies variants, and the like which possess *in vivo* stimulatory effects of human VEGF-D are all contemplated as being encompassed by the present invention.

In yet another embodiment, the invention provides a therapeutic or prophylactic method of treatment for lymphedema, comprising the step of administering to a mammalian subject in need of treatment for lymphedema a composition comprising a VEGF-C polypeptide, in an amount effective to treat or prevent swelling associated with lymphedema. Administration via one or more intravenous or subcutaneous injections is contemplated.

The VEGFR-3 allelic variant polynucleotides and polypeptides described herein that were discovered and characterized by the present inventors are themselves considered aspects of the invention. Such polynucleotides and polypeptides are useful, for example, in screening assays to study the biological activities of VEGFR-3 variant alleles and identify compounds that are capable of modulating that activity, *e.g.*, to identify therapeutic candidates for treatment of lymphedema. Such screening assays are also considered aspects of the invention.

The polypeptides of the invention are intended to include complete VEGFR-3 polypeptides with signal peptide (e.g., approximately residues 1 to 20 of SEQ ID NO: 2), mature VEGFR-3 polypeptides lacking any signal peptide, and recombinant variants wherein a foreign or synthetic signal peptide has been fused to

the mature VEGFR-3 polypeptide. Polynucleotides of the invention include all polynucleotides that encode all such polypeptides. It will be understood that for essentially any polypeptide, many polynucleotides can be constructed that encode the polypeptide by virtue of the well known degeneracy of the genetic code. All such polynucleotides are intended as aspects of the invention.

Thus, in yet another aspect, the invention provides a purified polynucleotide comprising a nucleotide sequence encoding a human VEGFR-3 protein variant, wherein said polynucleotide is capable of hybridizing to the complement of SEQ ID NO: 1 under stringent hybridization conditions, and wherein the encoded VEGFR-3 protein variant has an amino acid sequence that differs at position 1114 from the amino acid sequence set forth in SEQ ID NO: 1. Exemplary conditions are as follows: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM Na•PO4, pH 6.8; and washing in 0.2X SSC at 55°C. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. [See Sambrook et al. (1989), *supra*, §§ 9.47-9.51.]

In a related embodiment, the invention provides a purified polynucleotide comprising a nucleotide sequence encoding a VEGFR-3 protein of a human that is affected with heritable lymphedema or other lymphatic disorder; wherein the polynucleotide is capable of hybridizing to the complement of SEQ ID NO: 1 under stringent hybridization conditions, and wherein the encoded polynucleotide has an amino acid sequence that differs from SEQ ID NO: 1 at at least one codon. It will be understood that conventional recombinant techniques can be used to isolate such polynucleotides from individuals affected with heritable lymphedema or their relatives. The wildtype VEGFR-3 cDNA sequence set forth in SEQ ID NO: 1 (or its complement, or fragments thereof) is used as a probe to identify and isolate VEGFR-3 sequences from nucleic acid derived from the individuals. Alternatively, PCR amplification primers based on the wildtype VEGFR-3 sequence are generated and used to amplify either VEGFR-3 genomic DNA or VEGFR-3 mRNA from the human subject. The resultant amplified genomic DNA or cDNA is sequenced to determine the variations that characterize the VEGFR-3 lymphedema allele of the individual. A preferred VEGFR-3 lymphedema allele is the P1114L allele described in detail herein.

In addition, the invention provides vectors that comprise the polynucleotides of the invention. Such vectors are useful for amplifying and expressing the VEGFR-3 proteins encoded by the polynucleotides. The invention further provides a host cell transformed or transfected with polynucleotides (including vectors) of the invention. In a preferred embodiment, the host cell expresses the encoded VEGFR-3 protein on its surface. Such host cells are useful in cell-based screening assays for identifying modulators that stimulate or inhibit signaling of the encoded VEGFR-3. Modulators that stimulate VEGFR-3 signaling have utility as therapeutics to treat lymphedemas, whereas modulators that are inhibitory have utility for treating hyperplastic lymphatic conditions mediated by the allelic variant VEGFR-3. In a preferred embodiment, host cells of the invention are co-transfected with both a wildtype and an allelic variant VEGFR-3 polynucleotide, such that the cells express both receptor types on their surface. Such host cells are preferred for simulating a heterozygous VEGFR-3 genotype of many individuals affected with lymphedema.

In yet another aspect, the invention provides a transgenic mouse characterized by a non-native VEGFR-3 allele that has been introduced into the mouse, and the transgenic progeny thereof. Preferred allelic variants include allelic variants that correlate with hereditary lymphedema in human subjects, such as an allelic variant wherein a P1114L missense mutation has been introduced into the murine VEGFR-3 gene, or wherein the human P1114L allelic variant has been substituted for a murine VEGFR-3 allele. Such mice are produced using standard methods. [See, *e.g.*, Hogan *et al.* (eds.), *Manipulating the Mouse Embryo*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1986).]

In yet another aspect, the invention provides assays for identifying modulators of VEGFR-3 signaling, particularly modulators of the signaling of allelic variants of VEGFR-3 that correlate with lymphatic disorders such as heritable lymphedema. For example, the invention provides a method for identifying a modulator of intracellular VEGFR-3 signaling, comprising the steps of: contacting a cell expressing at least one mutant mammalian VEGFR-3 polypeptide in the presence and in the absence of a putative modulator compound; b) detecting VEGFR-3 signaling in the cell; and c) identifying a putative modulator compound in view of decreased or increased signaling in the presence of the putative modulator, as compared to signaling in the absence of the putative modulator.

By "mutant mammalian VEGFR-3 polypeptide" is meant a VEGFR-3 polypeptide that varies from a wildtype mammalian VEGFR-3 polypeptide (e.g., by virtue of one or more amino acid additions, deletions, or substitutions), wherein the variation is reflective of a naturally occurring variation that has been correlated with a lymphatic disorder, such as lymphedema. By way of example, a P1114L substitution variation at position 1114 (SEQ ID NO: 1) of human VEGFR-3 has been correlated with heritable lymphedema. The P1114L human allelic variant, or an analogous human allelic variant having a different substitution at position 1114, or a non-human VEGFR-3 into which a mutation at the position corresponding to P1114 has been introduced are all examples of mutant mammalian VEGFR-3 polypeptides.

The detecting step can entail the detection of any parameter indicative of VEGFR-3 signaling. For example, the detecting step can entail a measurement of VEGFR-3 autophosphorylation, or a measurement of VEGFR-3-mediated cell growth, or a measurement of any step in the VEGFR-3 signaling cascade between VEGFR-3 autophosphorylation and cell growth.

In a preferred embodiment, the method is practiced with a cell that expresses the mutant mammalian VEGFR-3 polypeptide and a wildtype mammalian VEGFR-3 polypeptide. Such cells are thought to better mimic the conditions in heterozygous individuals suffering from a VEGFR-3-mediated lymphatic disorder. In a highly preferred embodiment, the mutant and wildtype VEGFR-3 polypeptides are human. In a very highly preferred embodiment, the mutant VEGFR-3 polypeptide comprises a leucine amino acid at the position corresponding to position 1114 of SEQ ID NO: 1.

Additional features and variations of the invention will be apparent to those skilled in the art from the entirety of this application, including the drawing and detailed description, and all such features are intended as aspects of the invention. Likewise, features of the invention described herein can be re-combined into additional embodiments that are also intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. Although the applicant(s) invented the full scope of the claims appended hereto, the claims appended hereto are not intended to encompass within their scope the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

BRIEF DESCRIPTION OF THE DRAWING

Figures 1A-1F depict pedigrees of six hereditary lymphedema families (Families 101, 106, 111, 135, 105, and 127, respectively) informative for linkage. Filled symbols represent individuals with clinically documented lymphedema. Crossed symbols represent individuals with an ambiguous phenotype. An ambiguous phenotype is defined as self-reported swelling of the limbs with no known cause, without a clinical diagnosis of lymphedema. Individuals of ambiguous phenotype were coded as disease status unknown for the linkage analysis. The proband in each family is indicated by an arrow.

Figure 2 is a graph summarizing VITESSE analysis of lymphedema families with markers localized to chromosome 5q34-q35. In the graph, filled circles represent analyses for Families 101, 105, 106, and 111; open boxes represent analyses for Families 101, 106, and 111; open circles represent the VEGFR-3 gene; and open triangles represent Family 135. The one LOD confidence interval lies completely within the interval flanked by markers D5S1353 and D5S408 and overlaps the most likely location of *Flt4* (*VEGFR-3*). Linkage is excluded for the entire region for family 135.

DETAILED DESCRIPTION OF THE INVENTION

Certain therapeutic aspects of the present invention involve the administration of Vascular Endothelial Growth Factor C or D polynucleotides and

polypeptides. The growth factor VEGF-C, as well as native human, non-human mammalian, and avian polynucleotide sequences encoding VEGF-C, and VEGF-C variants and analogs, have been described in detail in International Patent Application Number PCT/US98/01973, filed 02 February 1998 and published on 06 August 1998 as International Publication Number WO 98/33917; in Joukov *et al., J. Biol. Chem., 273(12)*: 6599-6602 (1998); and in Joukov *et al., EMBO J., 16(13)*: 3898-3911 (1997), all of which are incorporated herein by reference in the entirety. As explained therein in detail, human VEGF-C is initially produced in human cells as a prepro-VEGF-C polypeptide of 419 amino acids. A cDNA and deduced amino acid sequence for human prepro-VEGF-C are set forth in SEQ ID NOs: 3 and 4, respectively, and a cDNA encoding human VEGF-C has been deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 (USA), pursuant to the provisions of the Budapest Treaty (Deposit date of 24 July 1995 and ATCC Accession Number 97231). VEGF-C sequences from other species have also been reported. See Genbank Accession Nos. MMU73620 (Mus musculus); and CCY15837 (Coturnix coturnix) for example, incorporated herein by reference.

The prepro-VEGF-C polypeptide is processed in multiple stages to produce a mature and most active VEGF-C polypeptide of about 21-23 kD (as assessed by SDS-PAGE under reducing conditions). Such processing includes cleavage of a signal peptide (SEQ ID NO: 4, residues 1-31); cleavage of a carboxyl-terminal peptide (corresponding approximately to amino acids 228-419 of SEQ ID NO: 4 and having a pattern of spaced cysteine residues reminiscent of a Balbiani ring 3 protein (BR3P) sequence [Dignam *et al., Gene, 88:*133-40 (1990); Paulsson *et al., J. Mol. Biol., 211:*331-49 (1990)]) to produce a partially-processed form of about 29 kD; and cleavage (apparently extracellularly) of an amino-terminal peptide (corresponding approximately to amino acids 32-103 of SEQ ID NO: 4) to produce a fully-processed mature form of about 21-23 kD. Experimental evidence demonstrates that partially-processed forms of VEGF-C (*e.g.*, the 29 kD form) are able to bind the VEGFR-3 receptor, whereas high affinity binding to VEGFR-2 occurs only with the fully processed forms of VEGF-C.

Moreover, it has been demonstrated that amino acids 103-227 of SEQ ID NO: 4 are not all critical for maintaining VEGF-C functions. A polypeptide consisting of amino acids 113-213 (and lacking residues 103-112 and 214-227) of

SEQ ID NO: 2 retains the ability to bind and stimulate VEGFR-3, and it is expected that a polypeptide spanning from about residue 131 to about residue 211 will retain VEGF-C biological activity. The cysteine residue at position 156 has been shown to be important for VEGFR-2 binding ability. However, VEGF-C ΔC_{156} polypeptides (i.e., analogs that lack this cysteine due to deletion or substitution) remain potent activators of VEGFR-3, and are therefore considered to be among the preferred candidates for treatment of lymphedema. The cysteine at position 165 of SEQ ID NO: 4 is essential for binding to either receptor, whereas analogs lacking the cysteines at positions 83 or 137 compete with native VEGF-C for binding with both receptors and are able to stimulate both receptors.

An alignment of human VEGF-C with VEGF-C from other species (performed using any generally accepted alignment algorithm) suggests additional residues wherein modifications can be introduced (*e.g.*, insertions, substitutions, and/or deletions) without destroying VEGF-C biological activity. Any position at which aligned VEGF-C polypeptides of two or more species have different amino acids, especially different amino acids with side chains of different chemical character, is a likely position susceptible to modification without concomitant elimination of function. An exemplary alignment of human, murine, and quail VEGF-C is set forth in Figure 5 of PCT/US98/01973.

Apart from the foregoing considerations, it will be understood that innumerable conservative amino acid substitutions can be performed to a wildtype VEGF-C sequence which are likely to result in a polypeptide that retains VEGF-C biological activities, especially if the number of such substitutions is small. By "conservative amino acid substitution" is meant substitution of an amino acid with an amino acid having a side chain of a similar chemical character. Similar amino acids for making conservative substitutions include those having an acidic side chain (glutamic acid, aspartic acid); a basic side chain (arginine, lysine, histidine); a polar amide side chain (glutamine, asparagine); a hydrophobic, aliphatic side chain (leucine, isoleucine, valine, alanine, glycine); an aromatic side chain (phenylalanine, tryptophan, tyrosine); a small side chain (glycine, alanine, serine, threonine, methionine); or an aliphatic hydroxyl side chain (serine, threonine). Addition or deletion of one or a few internal amino acids without destroying VEGF-C biological activities also is contemplated.

Without intending to be limited to a particular theory, the mechanism behind the efficacy of VEGF-C in treating or preventing lymphedema is believed to relate to the ability of VEGF-C to stimulate VEGFR-3 signaling. Administration of VEGF-C in quantities exceeding those usually found in interstitial fluids is expected to stimulate VEGFR-3 in human subjects who, by virtue of a dominant negative heterozygous mutation, have insufficient VEGFR-3 signaling.

The growth factor named Vascular Endothelial Growth Factor D (VEGF-D), as well as human sequences encoding VEGF-D, and VEGF-D variants and analogs, have been described in detail in International Patent Application Number PCT/US97/14696, filed 21 August 1997 and published on 26 February 1998 as International Publication Number WO 98/07832; and in Achen, *et al., Proc. Nat'l Acad. Sci. U.S.A., 95(2)*: 548-553 (1998), both incorporated herein by reference in the entirety. As explained therein in detail, human VEGF-D is initially produced in human cells as a prepro-VEGF-D polypeptide of 354 amino acids. A cDNA and deduced amino acid sequence for human prepro-VEGF-D are set forth in SEQ ID Nos: 5 and 6, respectively. VEGF-D sequences from other species also have been reported. See Genbank Accession Nos. D89628 (Mus musculus); and AF014827 (Rattus norvegicus), for example, incorporated herein by reference.

The prepro-VEGF-D polypeptide has a putative signal peptide of 21 amino acids and is apparently proteolytically processed in a manner analogous to the processing of prepro-VEGF-C. A "recombinantly matured" VEGF-D lacking residues 1-92 and 202-354 of SEQ ID NO: 6 retains the ability to activate receptors VEGFR-2 and VEGFR-3, and appears to associate as non-covalently linked dimers. Thus, preferred VEGF-D polynucleotides include those polynucleotides that comprise a nucleotide sequence encoding amino acids 93-201 of SEQ ID NO: 6.

The subject matter of the invention is further described and demonstrated with reference to the following examples.

EXAMPLE 1

Demonstration that hereditary lymphedema is linked to the VEGFR-3 locus

The following experiments, conducted to identify a gene or genes contributing to susceptibility to develop lymphedema, demonstrated that hereditary

lymphedema correlates, in at least some families, to the chromosomal locus for the VEGFR-3 gene.

OVERVIEW

Families with inherited lymphedema were identified for the purpose of conducting a linkage and positional candidate gene analysis. Thirteen distinct families from the United States and Canada were identified through referrals from lymphedema treatment centers, lymphedema support groups, and from internet correspondence (worldwide web site at www.pitt.edu/~genetics/lymph/) The study protocol was approved by the Institutional Review Board of the University of Pittsburgh and participants gave written informed consent. All members of the families were of western European ancestry. Forty members of one family ("Family 101") were examined during a family reunion by a physiatrist experienced in lymphedema treatment. Family members were considered affected with hereditary lymphedema if they exhibited asymmetry or obvious swelling of one or both legs. Members of the other 12 families were scored as affected if they had received a medical diagnosis of lymphedema, or if there were personal and family reports of extremity swelling or asymmetry. Medical records were obtained to verify status whenever possible. For the purpose of linkage analysis, individuals with very mild or intermittent swelling, heavyset legs, obesity, or a history of leg infections as the only symptom were considered to have indeterminate disease status.

In the 13 families, 105 individuals were classified as affected, with a male:female ratio of 1:2.3. The age of onset of lymphedema symptoms ranged from prenatal (diagnosed by ultrasound) to age 55. When affected by normal matings were analyzed, 76 of 191 children were affected, yielding a penetrance of 80%. First degree relatives of affected individuals were considered at risk.

Biological samples were obtained from members of the thirteen families to conduct the genetic analyses. DNA was isolated from the EDTA-anticoagulated whole blood by the method of Miller *et al.*, *Nucleic Acids Res., 16:* 1215 (1998), and from cytobrush specimens using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Analysis of the markers used in the genome scan were performed by methods recognized in the art. [See Browman *et al.*, *Am. J. Hum. Genetic., 63*:861-869 (1998); see also the NHLBI Mammalian Genotyping Service world-wide web sites

(www.marshmed.org/genetics/methods/pcr.htm; and www.marshmed.org/genetics/methods/gel.htm).

Two-point linkage analysis was conducted using an autosomal dominant model predicting 80% penetrance in the heterozygous state, 99% penetrance in the homozygous state, and a 1% phenocopy rate. The frequency of the disease allele was set at 1/10,000. Microsatellite marker allele frequencies were calculated by counting founder alleles, with the addition of counts of non-transmitted alleles. Multipoint analysis was carried out using distances obtained from the Location Database (LDB-http://cedar.genetics.soton.ac.uk/public html). Multipoint and 2-point analyses were facilitated using the VITESSE (v1.1) program. [O'Connell, J.R. and Weeks, D.E., (1995), *Nature Genet., 11:*402-408].

DETAILED DESCRIPTION OF METHODS AND RESULTS

The first family studied, Family 101, was a large, multi-generational family demonstrating early onset lymphedema. (See Fig. 1.) Forty individuals of this family were examined and DNA sampled. In addition, blood was obtained from another 11 members from mailing kits. Linkage simulation was performed using SLINK [Weeks *et al.*, *Am. J. Hum. Genet. 47:*A204 (1990)] and linkage was analyzed using MSIM [Ott, J., *Proc. Nat. Acad. Sci. USA, 86:*4175-4178 (1989)] to estimate the potential power of two point linkage analysis in the family. Marker genotypes were simulated for a marker with heterozygosity of 0.875 under a linked (θ=0) and unlinked (θ=0.5) model using the 51 available individuals. The simulation showed that the power to detect linkage was greater than 90% for a LOD score threshold of $Z(\theta)$ 2.0. The false positive rate was less than 5%.

Shortly thereafter, two additional families (designated Families 106 and 111) segregating for autosomal dominant lymphedema were identified. These three families (Figures 1A-1C, Families 101, 106 and 111) were genotyped for 366 autosomal markers by the NHLBI Mammalian Genotyping Service (www.marshmed.org/genetics). Genotypes were checked for consistency using Pedcheck [O'Connell, J.R. and Weeks, D.E., *Am. J. Hum. Genet., 61:*A288 (1997)]. Two point linkage analysis was performed using VITESSE [O'Connell, J.R. and Weeks, D.E., *Nature Genet., 11:*402-408 (1995)]. The model for linkage assumed an

autosomal dominant model of inheritance, a disease allele frequency of 0.0001 and a penetrance of 0.80.

The results from the genomic scan can be briefly summarized as follows. A summed LOD score of greater than 4.0 was observed from distal chromosome 5, markers *D5S1456*, *D5S817* and *D5S488*. The markers on distal chromosome 5q were the only markers having Z>3.0, the criteria established for statistical significance. LOD scores greater than 2.0 (θ=0-0.15) were also detected for chromosome 12 (*D12S391* Z=2.03, all families), and chromosome 21 (*D21S1440* Z=2.62, all families). The largest two-point LOD (Z=4.3; θ=0) was observed for marker *D5S408*, localized to chromosome 5q34-q35.

This initial chromosomal mapping was further refined by genotyping the three affected families for eight additional markers localized to region 5q34-q35. Six of these were informative for linkage (*D5S653*, *D5S498*, *D5S408*, *D5S2006*, *D5S1353* and *D5S1354*). Linkage analysis of these markers using VITESSE yielded a 2-point LOD score of 6.1 at θ=0 for marker *D5S1354* (Table 1) and a maximum multipoint LOD score of 8.8 at marker *D5S1354* (Fig. 2). These findings supported the localization of a gene within chromosome band 5q34-q35 that is a predisposing factor in hereditary lymphedema.

TABLE 1

LOD scores for individual families estimated over the interval defined by markers *D5S498* and *D5S2006*.

	Z(θ) 0.0	Z(θ) 0.01	Z(θ)0.05	Z(θ) 0.1	Z(θ) 0.2
Locus D5S498					
Family 101	-3.18	-2.33	-0.45	0.42	0.88
Family 106	1.08	1.07	1.05	0.99	0.81
Family 111	-0.85	-0.77	-0.53	-0.34	-0.13
Family 105	1.22	1.20	1.11	0.98	0.72
Family 135	-2.48	-1.85	-1.12	-0.75	-0.38

	Z(θ) 0.0	Z(θ) 0.01	Z(θ)0.05	Z(θ) 0.1	Z(θ) 0.2
Locus D5S1353					
Family 101	-2.99	-2.48	-1.21	-0.63	-0.18
Family 106	0.28	0.29	0.35	0.38	0.38
Family 111	-1.06	-1.02	-0.88	-0.72	-0.42
Family 105	0.72	0.71	0.65	0.56	0.39
Family 135	-8.03	-4.18	-2.09	-1.13	-0.30
Locus D5S1354					
Family 101	6.09	6.02	5.69	5.21	4.07
Family 106	1.42	1.40	1.32	1.20	0.96
Family 111	0.21	0.22	0.23	0.24	0.22
Family 105	0.43	0.42	0.40	0.36	0.28
Family 135	-6.88	-4.91	-3.20	-2.16	-1.07
Locus D5S408					
Family 101	2.80	2.74	2.50	2.20	1.56
Family 106	0.66	0.68	0.73	0.76	0.71
Family 111	-1.70	-1.40	-0.80	-0.44	-0.10
Family 105	0.42	0.41	0.38	0.35	0.27
Family 135	-5.22	-4.24	-2.58	-1.67	-0.80
Locus D5S2006					
Family 101	4.51	4.70	4.85	4.66	3.80
Family 106	1.17	1.16	1.11	1.03	0.83
Family 111	-1.32	-1.18	-0.82	-0.56	-0.25
Family 105	0.43	0.42	0.40	0.36	0.28
Family 135	-3.86	-3.20	-2.11	-1.45	-0.73

During the completion of the genome scan, an additional ten lymphedema families were ascertained. Two of these families (Families 105 and 135, see Figures 1E and 1D), were potentially informative for linkage and were genotyped for markers in the linked region. Examination of the two point LOD scores for the five informative families for markers in the linked region (Table 1) shows that four of the

families (101, 105, 106 and 111) are consistent with linkage to chromosome 5q while family 135 excluded linkage across the entire region with LOD scores Z =< -2.0 for all markers. Multipoint linkage analysis of Families 101, 105, 106 and 111 (Fig. 2) yielded a peak LOD score of Z = 10 at marker *D5S1354*. These findings support the existence of at least two loci which predispose to hereditary lymphedema.

The order of markers *D5S1353*, *D5S1354* and *D5S408* with respect to each other was uncertain. Multipoint linkage analysis using alternative orders for these markers gave similar results. Marker *D5S498* is a framework marker and marker *D5S408* is mapped 11.2 centimorgans distal to *D5S498*, based on the CHLC chromosome 5 sex averaged, recombination minimized map, version 3 (www.chlc.org). The physical distance between *D5S498* and *D5S408* is estimated as 1.45 megabases based on the Genetic Location Database (LDB) chromosome 5 summary map (cedar.genetics.soton.ac.uk/public_html/).

Database analysis identified sixteen genes within this region. Two of these genes have been identified as having roles in development (*MSX2* and VEGFR-3). *MSX2* was considered an unlikely candidate gene for lymphedema because of its known involvement in craniofacial development [Jabs *et al.*, *Cell, 75:* 443-450 (1993)]. VEGFR-3, the gene encoding a receptor for VEGF-C, was selected as a better candidate gene for initial further study for the following reasons.

(1) VEGFR-3 is expressed in developing lymphatic endothelium in the mouse [Kukk *et al.*, *Development, 122*: 3829-3837 (1996); and Kaipainen *et al.*, *Proc. Nat. Acad. Sci. USA, 92*: 3566-3570 (1995)];

(2) expression of VEGFR-3 is induced in differentiating avian chorioallantoic membrane [Oh *et al.*, *Dev. Biol., 188:*96-109 (1997)]; and

(3) overexpression of VEGF-C, a ligand of VEGFR-3, leads to hyperplasia of the lymphatic vessels in transgenic mice [Jeltsch *et al.*, *Science, 276:* 1423-1425 (1997)].

To explore the potential role of VEGFR-3 in lymphedema, probands from the thirteen lymphedema families were screened for variation by direct sequencing of portions of the VEGFR-3 gene. The sequencing strategy used amplification primers generated based upon the *VEGFR-3* cDNA sequence (SEQ ID NO: 1) and information on the genomic organization of the related vascular endothelial

growth factor receptor-2 (*VEGFR-2/KDR/flk-1*) [Yin *et al.*, *Mammalian Genome, 9:* 408-410 (1998)]. Variable positions (single nucleotide polymorphisms), the unique sequence primers used to amplify sequences flanking each variable site, and the method of detecting each variant are summarized in Table 2.

TABLE 2

Location, amplification primer sequences, amplification conditions, and detection methods for five intragenic single nucleotide polymorphisms in the human VEGFR-3 gene

Position in VEGFR-3 gene	Primer 1 sequence	Primer 2 sequence	Ann. temp.	[$MgCl_2$]	Base change	Detection Method
Exon 12, amino acid 641	tcaccatcgatccaagc (SEQ ID NO: 7)	agttctgcgtgagccgag (SEQ ID NO: 8)	56 °C	1.0 mM	C→T	Sequencing
Exon 24, amino acid 1114	caggacggggtgacttga (SEQ ID NO: 9)	gcccaggcctgtctactg (SEQ ID NO: 10)	56 °C	1.0 mM	C→T	Sequencing
Exon 3, amino acid 175	ccagctcctacgtgttcg (SEQ ID NO: 11)	ggcaacagctggatgtca (SEQ ID NO: 12)	56 °C	1.0 mM	C→T	*HhaI*
65bp 3' to Exon 6	ctgtgagggcgtgggagt (SEQ ID NO: 13)	gtcctttgagccactgga (SEQ ID NO: 14)	54 °C	1.5 mM	G→A	*StyI*
55bp 3' to Exon 2	cacacgtcatcgacaccggtg (SEQ ID NO: 15)	ggcaacagctggatgtca (SEQ ID NO: 16)	56 °C	1.5 mM	C→T	*ApaI*

All amplifications were done for 35 cycles with denaturation at 94° for 30 seconds, annealing as above for 30 seconds, and extension at 72° for 30 seconds.

Amplification and sequencing primers were synthesized by the DNA Synthesis Facility, University of Pittsburgh. Amplification primers were tagged at the 5' end with the forward or reverse M13 universal sequence to facilitate direct sequencing. Amplimers were subjected to cycle sequencing using the dRhodamine terminator ready reaction kit or the Dye Primer ready reaction kit for -M13 and M13 Rev primers (Perkin Elmer) and analyzed on the Prism ABI 377 fluorescent sequencer. Sequences were aligned for further analysis using SEQUENCHER 3.0 (Gene Codes).

Genomic sequence from approximately 50% of the VEGFR-3 gene was determined in this manner, and five single nucleotide variants were observed. Two of the variants occurred in introns, and a third was a silent substitution in predicted exon 3. These intragenic polymorphisms were used to map the *VEGFR-3* gene. As shown in Figure 2, *VEGFR-3* maps within the region of chromosome 5q linked to the lymphedema phenotype, consistent with it being selected as a candidate gene. In two

families, (Family 127, pedigree not shown, and Family 135), a C→T transition was identified at nucleotide position 1940 of the *VEGFR-3* cDNA (SEQ ID NO: 1). This nucleotide substitution is predicted to lead to a non-conservative substitution of serine (codon TCC) for proline (codon CCC) at residue 641 (putative exon 12, within the sixth immunoglobulin-like region of the receptor's extracellular domain) of the amino acid sequence of the receptor (SEQ ID NO: 2). However, this sequence change was observed in 2 of 120 randomly selected individuals from the general population (240 alleles). Also, in one of the two families in which this variant was initially detected, family 135, linkage between lymphedema and chromosome 5q markers was excluded (Table 1 and Figure 2). In probands from the other ten families, wild type sequence was observed at nucleotide position 1940. Collectively, these results suggest that this P641S variant is not causative.

In one nuclear family (Family 127, pedigree shown in Figure 1F) a C→T transition was observed at nucleotide position 3360 (SEQ ID NO: 1) of the *VEGFR-3* cDNA. This nucleotide substitution is predicted to lead to a non-conservative substitution of leucine (codon CTG) for proline (codon CCG) at residue 1114 of the amino acid sequence of the receptor (SEQ ID NO: 2). This P1114L mutation is predicted to lie in the intracellular tyrosine kinase domain II involved in intracellular signaling [Pajusola *et al.*, *Cancer Res.*, *52*:5738- 5743 (1992)]. Direct sequencing of predicted exon 20 of the *VEGFR-3* gene alleles from members of this family identified this substitution only in affected and at-risk family members. This sequence change was not observed in 120 randomly selected individuals of mixed European ancestry from the general population (240 alleles). In probands from the other 11 families, wild type sequence was observed at nucleotide position 3360.

Collectively, this data demonstrates that a missense mutation that causes a non-conservative substitution in a kinase domain of theVEGFR-3 protein correlates strongly with a heretable lymphedema in one family, and suggests that other mutations in the same gene may exist that correlate with heretable lymphedema in other families. As explained above, only a portion of the *VEGFR-3* gene sequence was analyzed to identify this first mutation of interest. Additional sequencing, using standard techniques and using the known *VEGFR-3* gene sequence for guidance, is expected to identify additional mutations of interest that are observed in affected and at-risk members of other families studied.

EXAMPLE 2

Demonstration that a C→T missense mutation at position 3360 in the VEGFR-3 coding sequence results in a tyrosine kinase negative mutant

The results set forth in Example 1 identified two missense mutations in the *VEGFR-3* coding sequence, one of which (C→T at position 3360) appeared to correlate with heritable lymphedema and one of which (C→T transition at position 1940) did not. The following experiments were conducted to determine the biochemical significance of these mutations on VEGFR-3 biological activity.

To analyze how the two single amino acid substitutions affect the VEGFR-3-mediated signaling, the corresponding mutant receptor expression vectors were generated using site-directed mutagenesis procedures and expressed in 293T cells by transient transfection. The long form of human VEGFR-3 cDNA (SEQ ID NO: 1) was cloned as a *Hind* III-*Bam* HI fragment from the LTR-FLT4l plasmid [Pajusola *et al., Oncogene 8*: 2931-2937 (1993)] into pcDNA3.1/Z(+) (Invitrogen). The P641S and P1114L mutants of VEGFR-3 were generated from this construct with the GeneEditor™ *in vitro* Site-Directed Mutagenesis System (Promega) using the following oligonucleotides (the C→T mutations are indicated with bold letters):

5'-CCTGAGTATC**T**CCCGCGTCGC-3' (SEQ ID NO: 17) for P641S mutation; and

5'-GGTGCCTCCC**T**GTACCCTGGG-3' (SEQ ID NO: 18) for P1114L mutation.

For the transient expression studies, 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO BRL, Life Technologies, Gaithersburg, MD), glutamine, and antibiotics. Cells were transfected with 20 μg of plasmid encoding the wild type or mutant VEGFR-3 forms using the calcium phosphate method, and harvested 36 hours after transfection for immunoprecipitation and Western blotting. Under these conditions, RTK overexpression results in ligand-independent activation, thus allowing the receptor phosphorylation to be studied. An empty vector was used for mock (control) transfections.

In order to investigate the effect of the two VEGFR-3 mutants on the tyrosine phosphorylation of the VEGFR-3, Western blotting analysis was performed using anti-phosphotyrosine antibodies. The cell monolayers were washed three times

with cold phosphate-buffered saline (PBS, containing 2 mM vanadate and 2 mM PMSF) and scraped into RIPA buffer (150 mM NaCl, 1 % Nonidet P40, 0.5 % deoxycholic acid sodium salt, 0.1 % SDS, 50 mM Tris-HCl, pH 8.0) containing 2 mM Vanadate, 2 mM PMSF, and 0.07 U/ml Aprotinin.

The cell lysates were sonicated and centrifuged for 10 minutes at 19,000 X g, and the supernatants were incubated for 2 hours on ice with 2 µg/ml of monoclonal anti-VEGFR-3 antibodies (9D9f9) [Jussila *et al.*, *Cancer Res., 58:* 1599-604 (1998)]. Thereafter, Protein A sepharose (Pharmacia) beads were added and incubation was continued for 45 minutes with rotation at +4°C. The sepharose beads were then washed three times with ice-cold RIPA buffer and twice with PBS (both containing 2 mM vanadate, 2 mM PMSF), analyzed by 7.5 % SDS-PAGE and transferred to a nitrocellulose filter (Protran Nitrocellulose, Schleicher & Schuell, No. 401196) using semi-dry transfer apparatus. After blocking the filter with 5 % BSA in TBS-T buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05 % Tween 20), the filters were incubated with the phosphotyrosine-specific primary antibodies (Upstate Biotechnology, #05-321), followed by biotinylated goat-anti-mouse immunoglobulins (Dako, E0433) and Biotin-Streptavidin HRP complex (Amersham, RPN1051). The bands were visualized by the enhanced chemiluminescence (ECL) method.

After analysis for phosphotyrine-containing proteins, the filters were stripped by washing for 30 minutes at +50°C in 100 mM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl, pH 6.7, with occasional agitation. The filters were washed with TBS-T, blocked again with BSA as described above, and analyzed for the presence of VEGFR-3 using the 9D9f9 antibodies and HRP-conjugated rabbit-anti-mouse immunoglobulins (Dako, P0161).

The Western analyses revealed that the P641S mutant receptor was phosphorylated normally, *i.e.*, in a manner similar to the wild type contol. However, the proteolytic processing of the P641S receptor protein may be affected, as the 175 kD and 125 kD polypeptides seemed to have a higher relative density when compared to the 195 kD form.

In contrast, no phosphorylated P1114L mutant protein was detected using the phosphotyrosine antibodies. The expression of similar amounts of the VEGFR-3 protein (normal and both mutants) was confirmed using the monoclonal 9D9f9 antibody, which is directed towards the extracellular domain of the VEGFR-3.

Both the P641S and the P1114L mutant VEGFR-3 migrated slightly faster than the wild type VEGFR-3 in the gel electrophoresis.

In order to analyze the possible dominant negative effect of the P1114L mutant on the wild-type receptor, a second, similar set of experiments were performed wherein the 293T cells were transfected with an increasing amount of the P1114L expression vector in combination with decreasing amounts of the wild type vector. Wild type to mutant ratios of 1:0, 3:1, 1:1, 1:3 and 0:1 were used. The cells were lysed 48 hours after transfection and the lysates were analyzed by immunoprecipitation and Western blotting as described above. These experiments permitted evaluation of whether the mutant protein interferes with wild type protein phosphorylation and estimation of the minimal amount of the WT protein needed for observeable tyrosyl autophosphorylation. Immunoprecipitates from cells transfected with only the WT plasmid revealed WT protein that was strongly phosphorylated in this experiment (lane 2), whereas immunoprecipitates from cells transfected with only the mutant plasmid were again inactive (unphosphorylated).

Interestingly, when transfection was made using 75% of WT and 25 % of mutant plasmid, the phosphorylation of the receptors was decreased by about 90%. This result strongly suggests that the P1114L mutant receptor forms heterodimers with the WT receptor, but cannot phosphorylate the WT receptor, thus failing to activate it. Under this theory, the WT receptor monomers in the heterodimers would also remain inactive, causing a disproportionate decrease of the total amount of activated receptor, when co-transfected with the mutant. Wildtype-wildtype homodimers would remain active and be responsible for the observed signaling. When the wild type and mutant receptor expression vectors were transfected at a 1:1 ratio, the VEGFR-3 phosphorylation was about 4% of the wild type alone, whereas at a 1:3 ratio, no tyrosine phosphorylation of VEGFR-3 was observed.

The foregoing results are consistent with the linkage analyses in Example 1: the mutation at position 641 that did not appear to correlate with lymphedema also did not appear to be disfunctional, whereas the mutation at position 1114 appeared to cause a dominant negative mutation that shows no tyrosine phosphorylation alone and that drastically reduces VEGFR-3 signalling in cells expressing both the mutant and wild type VEGFR-3 genes.

Collectively, these data indicate that the P1114L VEGFR-3 mutant is unable to act as a part of the signaling cascade, and also acts in a dominant negative manner, thus possibly interfering partially with the activation of the wild type VEGFR-3. Such effects of the mutation may eventually lead to lymphedema.

EXAMPLE 3

Treatment of lymphedema with a VEGFR-3 ligand

The data from Examples 1 and 2 collectively indicate a causative role in heritable lymphedema for a mutation in the *VEGFR-3* gene that interferes with VEGFR-3 signaling. Such a mutation behaves in an autosomal dominant pattern, due to the apparent necessity for receptor dimerization in the signaling process. However, the data from Example 2 suggests that some residual signaling may still occur in heterozygous affected individuals, presumably through pairing of VEGFR-3 proteins expressed from the wild type allele. The following experiments are designed to demonstrate the efficacy of VEGFR-3 ligand treatment in such affected individuals, to raise VEGFR-3 signaling to levels approaching normal and thereby ameliorate/palliate the symptoms of hereditary lymphedema.

Initially, an appropriate animal model is selected. Several potential animal models have been described in the literature. [See, e.g., Lyon *et al., Mouse News Lett. 71*: 26 (1984), *Mouse News Lett. 74*: 96 (1986), and *Genetic variants and strains of the laboratory mouse*, 2nd ed., New York: Oxford University Press (1989), p. 70 (*Chylous ascites* mouse); Dumont *et al., Science, 282*: 946-949 (1998) (heterozygous VEGFR-3 knockout mouse); Patterson *et al.*, "Hereditary Lymphedema," *Comparative Pathology Bulletin, 3*: 2 (1971) (canine hereditary lymphedema model); van der Putte, "Congenital Hereditary Lymphedema in the Pig," *Lympho, 11*: 1-9 (1978); and Campbell-Beggs *et al.*, "Chyloabdomen in a neonatal foal," *Veterinary Record, 137*: 96-98 (1995).] Those models which are determined to have analogous mutations to the VEGFR-3 gene are preferred. In a preferred embodiment, "knock in" homologous recombination genetic engineering strategies are used to create an animal model (*e.g.*, a mouse model) having a VEGFR-3 allelic variation analogous to the human variations described herein. [See, *e.g.*, Partanen *et al., Genes & Development, 12*: 2332-2344 (1998) (gene targeting to introduce mutations into another receptor protein (FGFR-1) in mice).] For example, the P1114L

mutation in human VEGFR-3 occurs in a VEGFR-3 region having highly conserved amino acid identity with murine VEGFR-3 (Genbank Accession No. L07296). Thus, a corresponding P1114L can be introduced into the murine VEGFR-3 by "knock-in" homologous recombination. Optionally, such mice can be bred to the heterozygous VEGFR-3 knockout mice or *Chy* mice described above to further modify the phenotypic severity of the lymphedema disease.

The mice as described above are treated with a candidate therapeutic, e.g., a recombinant mature form of VEGF-C, at various dosing schedules, *e.g.*, once daily by intravenous (IV) or intramuscular (IM) injection at a dose of 1-1000 ng/g body weight, preferably 10-100 ng/g, which should result in a peak level saturating VEGFR-3 (K_d about 150 pM) but not VEGFR-2 (K_d around 400 pM). Direct IM injection at multiple sites in the muscles of affected extremities is a preferred route of administration. The dosing is adjusted according to the efficacy of the treatment and the presence of possible side effects due to the lowering of blood pressure, which has been observed in response to VEGF administration IV. The efficacy of treatment is measured via NMRI imaging of the water content and volume of swelling of the abdomen and the extremities of the animals. The amount of fluid in the abdominal cavity is estimated and the animals are weighed during the follow-up.

In studies using VEGFR-3 -/+ x *Chy* mice progeny, the animals will also have the ß-galactosidase marker in their lymphatic endothelium. After a successful treatment, the treated and non-treated experimental animals and VEGFR-3 -/+ controls are killed and their lymphatic vessels are visualized by ß-gal and antibody staining. The staining patterns of experimental and control animals are compared for vessel diameter, numbers of endothelial cells, density of blood and lymphatic vessels, and nuclear density/section surface area for the estimation of tissue oedema.

Such experiments are repeated with various candidate therapeutics (e.g., VEGF-C or VEGF-D recombinant polypeptides; VEGF-C and VEGF-D gene therapy vectors; and combinations thereof) at various dosing schedules to determine an optimum treatment regimen.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art, all of which are intended as aspects of the present invention. Accordingly, only such limitations as appear in the claims should be placed on the invention.

CLAIMS

What is claimed is:

1. A method of screening a human subject for an increased risk of developing a lymphatic disorder, comprising the steps of:

(a) assaying nucleic acid of a human subject to determine a presence or an absence of a mutation altering the sequence or expression of at least one VEGFR-3 allele; and

(b) screening for an increased risk of developing a lymphatic disorder from the presence or absence of said mutation, wherein the presence of a mutation altering the encoded amino acid sequence or expression of at least one VEGFR-3 allele in the nucleic acid correlates with an increased risk of developing a lymphatic disorder.

2. A method according to claim 1 wherein the assaying step comprises determining the presence or absence of a mutation altering a tyrosine kinase domain amino acid sequence of the protein encoded by the VEGFR-3 allele.

3. A method according to claim 1 wherein the assaying step comprises determining the presence or absence of a missense mutation in the VEGFR-3 allele at a position corresponding to codon 1114 of the VEGFR-3 amino acid sequence set forth in SEQ ID NO: 2.

4. A method according to claim 1 wherein the assaying step comprises at least one procedure selected from the group consisting of:

(a) determining a nucleotide sequence of at least one codon of at least one VEGFR-3 allele of the human subject;

(b) performing a hybridization assay to determine whether nucleic acid from the human subject has a nucleotide sequence identical to or different from one or more reference sequences;

(c) performing a polynucleotide migration assay to determine whether nucleic acid from the human subject has a nucleotide sequence identical to or different from one or more reference sequences; and

(d) performing a restriction endonuclease digestion to determine whether nucleic acid from the human subject has a nucleotide sequence identical to or different from one or more reference sequences.

5. A method according to claim 1 wherein the assaying step comprises: performing a polymerase chain reaction (PCR) to amplify nucleic acid comprising VEGFR-3 coding sequence, and determining nucleotide sequence of the amplified nucleic acid.

6. A method of screening for a VEGFR-3 hereditary lymphedema genotype in a human patient, comprising the steps of:

(a) providing a biological sample comprising nucleic acid from said patient, said nucleic acid including sequences corresponding to said patient's VEGFR-3 alleles;

(b) analyzing said nucleic acid for the presence of a mutation or mutations;

(c) determining a VEGFR-3 genotype from said analyzing step; and

(d) correlating the presence of a mutation in a VEGFR-3 allele with a hereditary lymphedema genotype.

7. The method according to claim 6 wherein said biological sample is a cell sample.

8. The method according to claim 6 wherein said analyzing comprises sequencing a portion of said nucleic acid, said portion comprising at least one codon of said VEGFR-3 alleles.

9. The method according to claim 8 wherein said nucleic acid is DNA.

10. The method according to claim 8 wherein said nucleic acid is RNA.

11. A method of treatment for hereditary lymphedema, comprising the step of administering to a patient with hereditary lymphedema a therapeutically effective amount of a growth factor product selected from the group consisting of vascular endothelial growth factor C (VEGF-C) protein products, vascular endothelial growth factor D (VEGF-D) protein products, VEGF-C gene therapy products, and VEGF-D gene therapy protein products.

12. A therapeutic or prophylactic method of treating lymphedema, comprising the steps of:

providing isolated lymphatic endothelial cells or lymphatic endothelial progenitor cells;

transforming or transfecting the cells *ex vivo* with a polynucleotide comprising a nucleotide sequence that encodes a wild type VEGFR-3;

and administering the transformed or transfected cells to the mammalian subject.

13. An oligonucleotide useful as a probe for identifying polymorphisms in a human Flt4 receptor tyrosine kinase gene, the oligonucleotide comprising 6-50 nucleotides that have a sequence that is identical or exactly complementary to a portion of a human VEGFR-3 gene sequence or VEGFR-3 coding sequence, except for one sequence difference selected from the group consisting of a nucleotide addition, a nucleotide deletion, or nucleotide substitution.

14. An oligonucleotide according to claim 13 wherein the nucleotide sequence is exactly identical or exactly complementary to a portion of the human VEGFR-3 coding sequence set forth in SEQ ID NO: 1, except for a nucleotide substitution at a position corresponding to nucleotide 3360 of SEQ ID NO: 1.

15. A kit comprising at least two oligonucleotides of the formula X_nYZ_m or its complement;

where n and m are integers from 0 to 49;

where $5 \leq (n + m) \leq 49$;

where X_n is a stretch of n nucleotides identical to a first portion of SEQ ID NO: 1, said first portion ending immediately upstream (5') of position 3360 of SEQ ID NO: 1; and

where Z_m is a stretch of m nucleotides identical to a second portion of SEQ ID NO: 1, said second portion beginning immediately downstream (3') of position 3360 of SEQ ID NO: 1; and

wherein Y represents a nucleotide selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil nucleotides.

16. An array of oligonucleotide probes immobilized on a solid support, wherein each probe occupies a separate known site in the array; and wherein the array includes at least one probe set comprising two to four probes, wherein one probe is exactly identical or exactly complementary to a human VEGFR-3 coding sequence, and the other one to three members of the set are exactly identical to the first member, but for at least one different nucleotide, which different nucleotide is located in the same position in each of the one to three additional set members.

17. A purified polynucleotide comprising a nucleotide sequence encoding a human VEGFR-3 protein variant, wherein said polynucleotide is capable of hybridizing to the complement of SEQ ID NO: 1 under the following hybridization conditions:

hybridization at 42°C in 50% formamide, 5X SSC, 20 mM Na•PO4, pH 6.8; and

washing in 0.2X SSC at 55°C;

and wherein the encoded VEGFR-3 protein variant has an amino acid sequence that differs at position 1114 from the amino acid sequence set forth in SEQ ID NO: 1.

18. A purified polynucleotide comprising a nucleotide sequence encoding a VEGFR-3 protein of a human that is affected with heritable lymphedema;

wherein said polynucleotide is capable of hybridizing to the complement of SEQ ID NO: 1 under the following hybridization conditions: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM Na•PO4, pH 6.8; and washing in 0.2X SSC at 55°C;

and wherein the encoded polynucleotide has an amino acid sequence that differs from SEQ ID NO: 1 at at least one codon.

19. A vector comprising a polynucleotide according to claim 18.

20. A host cell that has been transformed or transfected with a polynucleotide according to claim 18 and that expresses the VEGFR-3 protein encoded by the polynucleotide.

21. A host cell according to claim 20 that has been co-transfected with a polynucleotide encoding the VEGFR-3 amino acid sequence set forth in SEQ ID NO: 2 and that expresses the VEGFR-3 protein having the amino acid sequence set forth in SEQ ID NO: 2.

22. A method for identifying a modulator of intracellular VEGFR-3 signaling, comprising the steps of:

a) contacting a cell expressing at least one mutant mammalian VEGFR-3 polypeptide in the presence and in the absence of a putative modulator compound;

b) detecting VEGFR-3 signaling in the cell; and

c) identifying a putative modulator compound in view of decreased or increased signaling in the presence of the putative modulator, as compared to signaling in the absence of the putative modulator.

23. A method according to claim 22 wherein the cell expresses the mutant mammalian VEGFR-3 polypeptide and a wildtype mammalian VEGFR-3 polypeptide.

24. A method according to claim 23 wherein the mutant and wildtype VEGFR-3 polypeptides are human.

25. A method according to claim 24 wherein the mutant VEGFR-3 polypeptide comprises a leucine amino acid at the position corresponding to position 1114 of SEQ ID NO: 1.

SEQUENCE LISTING

<110> Ferrell, Robert E
Alitalo, Kari
Finegold, David N
Karkkainen, Marika

<120> SCREENING AND THERAPY FOR LYMPATIC DISORDERS INVOLVING THE FLT4 RECEPTOR TYROSINE KINASE (VEGFR-3)

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Trp Leu Cys Leu Gly Leu Leu Asp Gly Leu Val Ser Gly Tyr Ser Met
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acc ccc ccg acc ttg aac atc acg gag gag tca cac gtc atc gac acc  148
Thr Pro Pro Thr Leu Asn Ile Thr Glu Glu Ser His Val Ile Asp Thr
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ggt gac agc ctg tcc atc tcc tgc agg gga cag cac ccc ctc gag tgg  196
Gly Asp Ser Leu Ser Ile Ser Cys Arg Gly Gln His Pro Leu Glu Trp
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gct tgg cca gga gct cag gag gcg cca gcc acc gga gac aag gac agc  244
Ala Trp Pro Gly Ala Gln Glu Ala Pro Ala Thr Gly Asp Lys Asp Ser
 60                  65                  70                  75

gag gac acg ggg gtg gtg cga gac tgc gag ggc aca gac gcc agg ccc  292
Glu Asp Thr Gly Val Val Arg Asp Cys Glu Gly Thr Asp Ala Arg Pro
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agc tac gtc tgc tac tac aag tac atc aag gca cgc atc gag ggc acc   388
Ser Tyr Val Cys Tyr Tyr Lys Tyr Ile Lys Ala Arg Ile Glu Gly Thr
        110                 115                 120

acg gcc gcc agc tcc tac gtg ttc gtg aga gac ttt gag cag cca ttc   436
Thr Ala Ala Ser Ser Tyr Val Phe Val Arg Asp Phe Glu Gln Pro Phe
    125                 130                 135

atc aac aag cct gac acg ctc ttg gtc aac agg aag gac gcc atg tgg   484
Ile Asn Lys Pro Asp Thr Leu Leu Val Asn Arg Lys Asp Ala Met Trp
140                 145                 150                 155

gtg ccc tgt ctg gtg tcc atc ccc ggc ctc aat gtc acg ctg cgc tcg   532
Val Pro Cys Leu Val Ser Ile Pro Gly Leu Asn Val Thr Leu Arg Ser
                160                 165                 170

caa agc tcg gtg ctg tgg cca gac ggg cag gag gtg gtg tgg gat gac   580
Gln Ser Ser Val Leu Trp Pro Asp Gly Gln Glu Val Val Trp Asp Asp
            175                 180                 185

cgg cgg ggc atg ctc gtg tcc acg cca ctg ctg cac gat gcc ctg tac   628
Arg Arg Gly Met Leu Val Ser Thr Pro Leu Leu His Asp Ala Leu Tyr
        190                 195                 200

ctg cag tgc gag acc acc tgg gga gac cag gac ttc ctt tcc aac ccc   676
Leu Gln Cys Glu Thr Thr Trp Gly Asp Gln Asp Phe Leu Ser Asn Pro
    205                 210                 215

ttc ctg gtg cac atc aca ggc aac gag ctc tat gac atc cag ctg ttg   724
Phe Leu Val His Ile Thr Gly Asn Glu Leu Tyr Asp Ile Gln Leu Leu
220                 225                 230                 235

ccc agg aag tcg ctg gag ctg ctg gta ggg gag aag ctg gtc ctg aac   772
Pro Arg Lys Ser Leu Glu Leu Leu Val Gly Glu Lys Leu Val Leu Asn
                240                 245                 250

tgc acc gtg tgg gct gag ttt aac tca ggt gtc acc ttt gac tgg gac   820
Cys Thr Val Trp Ala Glu Phe Asn Ser Gly Val Thr Phe Asp Trp Asp
            255                 260                 265

tac cca ggg aag cag gca gag cgg ggt aag tgg gtg ccc gag cga cgc   868
Tyr Pro Gly Lys Gln Ala Glu Arg Gly Lys Trp Val Pro Glu Arg Arg
        270                 275                 280

tcc cag cag acc cac aca gaa ctc tcc agc atc ctg acc atc cac aac   916
Ser Gln Gln Thr His Thr Glu Leu Ser Ser Ile Leu Thr Ile His Asn
    285                 290                 295

gtc agc cag cac gac ctg ggc tcg tat gtg tgc aag gcc aac aac ggc   964
Val Ser Gln His Asp Leu Gly Ser Tyr Val Cys Lys Ala Asn Asn Gly
300                 305                 310                 315
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atc cag cga ttt cgg gag agc acc gag gtc att gtg cat gaa aat ccc   1012
Ile Gln Arg Phe Arg Glu Ser Thr Glu Val Ile Val His Glu Asn Pro
                320                 325                 330

ttc atc agc gtc gag tgg ctc aaa gga ccc atc ctg gag gcc acg gca   1060
Phe Ile Ser Val Glu Trp Leu Lys Gly Pro Ile Leu Glu Ala Thr Ala
            335                 340                 345

gga gac gag ctg gtg aag ctg ccc gtg aag ctg gca gcg tac ccc ccg   1108
Gly Asp Glu Leu Val Lys Leu Pro Val Lys Leu Ala Ala Tyr Pro Pro
        350                 355                 360

ccc gag ttc cag tgg tac aag gat gga aag gca ctg tcc ggg cgc cac   1156
Pro Glu Phe Gln Trp Tyr Lys Asp Gly Lys Ala Leu Ser Gly Arg His
    365                 370                 375

agt cca cat gcc ctg gtg ctc aag gag gtg aca gag gcc agc aca ggc   1204
Ser Pro His Ala Leu Val Leu Lys Glu Val Thr Glu Ala Ser Thr Gly
380                 385                 390                 395

acc tac acc ctc gcc ctg tgg aac tcc gct gct ggc ctg agg cgc aac   1252
Thr Tyr Thr Leu Ala Leu Trp Asn Ser Ala Ala Gly Leu Arg Arg Asn
                400                 405                 410

atc agc ctg gag ctg gtg gtg aat gtg ccc ccc cag ata cat gag aag   1300
Ile Ser Leu Glu Leu Val Val Asn Val Pro Pro Gln Ile His Glu Lys
            415                 420                 425

gag gcc tcc tcc ccc agc atc tac tcg cgt cac agc cgc cag gcc ctc   1348
Glu Ala Ser Ser Pro Ser Ile Tyr Ser Arg His Ser Arg Gln Ala Leu
        430                 435                 440

acc tgc acg gcc tac ggg gtg ccc ctg cct ctc agc atc cag tgg cac   1396
Thr Cys Thr Ala Tyr Gly Val Pro Leu Pro Leu Ser Ile Gln Trp His
    445                 450                 455

tgg cgg ccc tgg aca ccc tgc aag atg ttt gcc cag cgt agt ctc cgg   1444
Trp Arg Pro Trp Thr Pro Cys Lys Met Phe Ala Gln Arg Ser Leu Arg
460                 465                 470                 475

cgg cgg cag cag caa gac ctc atg cca cag tgc cgt gac tgg agg gcg   1492
Arg Arg Gln Gln Gln Asp Leu Met Pro Gln Cys Arg Asp Trp Arg Ala
                480                 485                 490

gtg acc acg cag gat gcc gtg aac ccc atc gag agc ctg gac acc tgg   1540
Val Thr Thr Gln Asp Ala Val Asn Pro Ile Glu Ser Leu Asp Thr Trp
            495                 500                 505

acc gag ttt gtg gag gga aag aat aag act gtg agc aag ctg gtg atc   1588
Thr Glu Phe Val Glu Gly Lys Asn Lys Thr Val Ser Lys Leu Val Ile
        510                 515                 520

cag aat gcc aac gtg tct gcc atg tac aag tgt gtg gtc tcc aac aag   1636
Gln Asn Ala Asn Val Ser Ala Met Tyr Lys Cys Val Val Ser Asn Lys
    525                 530                 535
```

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gtg ggc cag gat gag cgg ctc atc tac ttc tat gtg acc acc atc ccc   1684
Val Gly Gln Asp Glu Arg Leu Ile Tyr Phe Tyr Val Thr Thr Ile Pro
540                 545                 550                 555

gac ggc ttc acc atc gaa tcc aag cca tcc gag gag cta cta gag ggc   1732
Asp Gly Phe Thr Ile Glu Ser Lys Pro Ser Glu Glu Leu Leu Glu Gly
                560                 565                 570

cag ccg gtg ctc ctg agc tgc caa gcc gac agc tac aag tac gag cat   1780
Gln Pro Val Leu Leu Ser Cys Gln Ala Asp Ser Tyr Lys Tyr Glu His
            575                 580                 585

ctg cgc tgg tac cgc ctc aac ctg tcc acg ctg cac gat gcg cac ggg   1828
Leu Arg Trp Tyr Arg Leu Asn Leu Ser Thr Leu His Asp Ala His Gly
        590                 595                 600

aac ccg ctt ctg ctc gac tgc aag aac gtg cat ctg ttc gcc acc cct   1876
Asn Pro Leu Leu Leu Asp Cys Lys Asn Val His Leu Phe Ala Thr Pro
    605                 610                 615

ctg gcc gcc agc ctg gag gag gtg gca cct ggg gcg cgc cac gcc acg   1924
Leu Ala Ala Ser Leu Glu Glu Val Ala Pro Gly Ala Arg His Ala Thr
620                 625                 630                 635

ctc agc ctg agt atc ccc cgc gtc gcg ccc gag cac gag ggc cac tat   1972
Leu Ser Leu Ser Ile Pro Arg Val Ala Pro Glu His Glu Gly His Tyr
                640                 645                 650

gtg tgc gaa gtg caa gac cgg cgc agc cat gac aag cac tgc cac aag   2020
Val Cys Glu Val Gln Asp Arg Arg Ser His Asp Lys His Cys His Lys
            655                 660                 665

aag tac ctg tcg gtg cag gcc ctg gaa gcc cct cgg ctc acg cag aac   2068
Lys Tyr Leu Ser Val Gln Ala Leu Glu Ala Pro Arg Leu Thr Gln Asn
        670                 675                 680

ttg acc gac ctc ctg gtg aac gtg agc gac tcg ctg gag atg cag tgc   2116
Leu Thr Asp Leu Leu Val Asn Val Ser Asp Ser Leu Glu Met Gln Cys
    685                 690                 695

ttg gtg gcc gga gcg cac gcg ccc agc atc gtg tgg tac aaa gac gag   2164
Leu Val Ala Gly Ala His Ala Pro Ser Ile Val Trp Tyr Lys Asp Glu
700                 705                 710                 715

agg ctg ctg gag gaa aag tct gga gtc gac ttg gcg gac tcc aac cag   2212
Arg Leu Leu Glu Glu Lys Ser Gly Val Asp Leu Ala Asp Ser Asn Gln
                720                 725                 730

aag ctg agc atc cag cgc gtg cgc gag gag gat gcg gga cgc tat ctg   2260
Lys Leu Ser Ile Gln Arg Val Arg Glu Glu Asp Ala Gly Arg Tyr Leu
            735                 740                 745

tgc agc gtg tgc aac gcc aag ggc tgc gtc aac tcc tcc gcc agc gtg   2308
Cys Ser Val Cys Asn Ala Lys Gly Cys Val Asn Ser Ser Ala Ser Val
        750                 755                 760
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gcc gtg gaa ggc tcc gag gat aag ggc agc atg gag atc gtg atc ctt   2356
Ala Val Glu Gly Ser Glu Asp Lys Gly Ser Met Glu Ile Val Ile Leu
    765                 770                 775

gtc ggt acc ggc gtc atc gct gtc ttc ttc tgg gtc ctc ctc ctc ctc   2404
Val Gly Thr Gly Val Ile Ala Val Phe Phe Trp Val Leu Leu Leu Leu
780                 785                 790                 795

atc ttc tgt aac atg agg agg ccg gcc cac gca gac atc aag acg ggc   2452
Ile Phe Cys Asn Met Arg Arg Pro Ala His Ala Asp Ile Lys Thr Gly
                800                 805                 810

tac ctg tcc atc atc atg gac ccc ggg gag gtg cct ctg gag gag caa   2500
Tyr Leu Ser Ile Ile Met Asp Pro Gly Glu Val Pro Leu Glu Glu Gln
            815                 820                 825

tgc gaa tac ctg tcc tac gat gcc agc cag tgg gaa ttc ccc cga gag   2548
Cys Glu Tyr Leu Ser Tyr Asp Ala Ser Gln Trp Glu Phe Pro Arg Glu
        830                 835                 840

cgg ctg cac ctg ggg aga gtg ctc ggc tac ggc gcc ttc ggg aag gtg   2596
Arg Leu His Leu Gly Arg Val Leu Gly Tyr Gly Ala Phe Gly Lys Val
    845                 850                 855

gtg gaa gcc tcc gct ttc ggc atc cac aag ggc agc agc tgt gac acc   2644
Val Glu Ala Ser Ala Phe Gly Ile His Lys Gly Ser Ser Cys Asp Thr
860                 865                 870                 875

gtg gcc gtg aaa atg ctg aaa gag ggc gcc acg gcc agc gag cac cgc   2692
Val Ala Val Lys Met Leu Lys Glu Gly Ala Thr Ala Ser Glu His Arg
                880                 885                 890

gcg ctg atg tcg gag ctc aag atc ctc att cac atc ggc aac cac ctc   2740
Ala Leu Met Ser Glu Leu Lys Ile Leu Ile His Ile Gly Asn His Leu
            895                 900                 905

aac gtg gtc aac ctc ctc ggg gcg tgc acc aag ccg cag ggc ccc ctc   2788
Asn Val Val Asn Leu Leu Gly Ala Cys Thr Lys Pro Gln Gly Pro Leu
        910                 915                 920

atg gtg atc gtg gag ttc tgc aag tac ggc aac ctc tcc aac ttc ctg   2836
Met Val Ile Val Glu Phe Cys Lys Tyr Gly Asn Leu Ser Asn Phe Leu
    925                 930                 935

cgc gcc aag cgg gac gcc ttc agc ccc tgc gcg gag aag tct ccc gag   2884
Arg Ala Lys Arg Asp Ala Phe Ser Pro Cys Ala Glu Lys Ser Pro Glu
940                 945                 950                 955

cag cgc gga cgc ttc cgc gcc atg gtg gag ctc gcc agg ctg gat cgg   2932
Gln Arg Gly Arg Phe Arg Ala Met Val Glu Leu Ala Arg Leu Asp Arg
                960                 965                 970

agg cgg ccg ggg agc agc gac agg gtc ctc ttc gcg cgg ttc tcg aag   2980
Arg Arg Pro Gly Ser Ser Asp Arg Val Leu Phe Ala Arg Phe Ser Lys
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acc gag ggc gga gcg agg cgg gct tct cca gac caa gaa gct gag gac   3028
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ctg tgg ctg agc ccg ctg acc atg gaa gat ctt gtc tgc tac agc ttc   3076
Leu Trp Leu Ser Pro Leu Thr Met Glu Asp Leu Val Cys Tyr Ser Phe
   1005                1010                1015

cag gtg gcc aga ggg atg gag ttc ctg gct tcc cga aag tgc atc cac   3124
Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His
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aga gac ctg gct gct cgg aac att ctg ctg tcg gaa agc gac gtg gtg   3172
Arg Asp Leu Ala Ala Arg Asn Ile Leu Leu Ser Glu Ser Asp Val Val
               1040                1045                1050

aag atc tgt gac ttt ggc ctt gcc cgg gac atc tac aaa gac cct gac   3220
Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp
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Tyr Val Arg Lys Gly Ser Ala Arg Leu Pro Leu Lys Trp Met Ala Pro
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gaa agc atc ttc gac aag gtg tac acc acg cag agt gac gtg tgg tcc   3316
Glu Ser Ile Phe Asp Lys Val Tyr Thr Thr Gln Ser Asp Val Trp Ser
   1085                1090                1095

ttt ggg gtg ctt ctc tgg gag atc ttc tct ctg ggg gcc tcc ccg tac   3364
Phe Gly Val Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr
1100                1105                1110                1115

cct ggg gtg cag atc aat gag gag ttc tgc cag cgg ctg aga gac ggc   3412
Pro Gly Val Gln Ile Asn Glu Glu Phe Cys Gln Arg Leu Arg Asp Gly
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aca agg atg agg gcc ccg gag ctg gcc act ccc gcc ata cgc cgc atc   3460
Thr Arg Met Arg Ala Pro Glu Leu Ala Thr Pro Ala Ile Arg Arg Ile
           1135                1140                1145

atg ctg aac tgc tgg tcc gga gac ccc aag gcg aga cct gca ttc tcg   3508
Met Leu Asn Cys Trp Ser Gly Asp Pro Lys Ala Arg Pro Ala Phe Ser
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gag ctg gtg gag atc ctg ggg gac ctg ctc cag ggc agg ggc ctg caa   3556
Glu Leu Val Glu Ile Leu Gly Asp Leu Leu Gln Gly Arg Gly Leu Gln
   1165                1170                1175

gag gaa gag gag gtc tgc atg gcc ccg cgc agc tct cag agc tca gaa   3604
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1180                1185                1190                1195

gag ggc agc ttc tcg cag gtg tcc acc atg gcc cta cac atc gcc cag   3652
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gcc agg tat tac aac tgg gtg tcc ttt ccc ggg tgc ctg gcc aga ggg   3748
Ala Arg Tyr Tyr Asn Trp Val Ser Phe Pro Gly Cys Leu Ala Arg Gly
      1230                1235                1240

gct gag acc cgt ggt tcc tcc agg atg aag aca ttt gag gaa ttc ccc   3796
Ala Glu Thr Arg Gly Ser Ser Arg Met Lys Thr Phe Glu Glu Phe Pro
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atg acc cca acg acc tac aaa ggc tct gtg gac aac cag aca gac agt   3844
Met Thr Pro Thr Thr Tyr Lys Gly Ser Val Asp Asn Gln Thr Asp Ser
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ggg atg gtg ctg gcc tcg gag gag ttt gag cag ata gag agc agg cat   3892
Gly Met Val Leu Ala Ser Glu Glu Phe Glu Gln Ile Glu Ser Arg His
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aga caa gaa agc ggc ttc agc tgt aaa gga cct ggc cag aat gtg gct   3940
Arg Gln Glu Ser Gly Phe Ser Cys Lys Gly Pro Gly Gln Asn Val Ala
          1295                1300                1305

gtg acc agg gca cac cct gac tcc caa ggg agg cgg cgg cgg cct gag   3988
Val Thr Arg Ala His Pro Asp Ser Gln Gly Arg Arg Arg Arg Pro Glu
      1310                1315                1320

cgg ggg gcc cga gga ggc cag gtg ttt tac aac agc gag tat ggg gag   4036
Arg Gly Ala Arg Gly Gly Gln Val Phe Tyr Asn Ser Glu Tyr Gly Glu
  1325                1330                1335

ctg tcg gag cca agc gag gag gac cac tgc tcc ccg tct gcc cgc gtg   4084
Leu Ser Glu Pro Ser Glu Glu Asp His Cys Ser Pro Ser Ala Arg Val
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Asn Ile Thr Glu Glu Ser His Val Ile Asp Thr Gly Asp Ser Leu Ser
        35                  40                  45
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Ile Ser Cys Arg Gly Gln His Pro Leu Glu Trp Ala Trp Pro Gly Ala
 50                  55                  60

Gln Glu Ala Pro Ala Thr Gly Asp Lys Asp Ser Glu Asp Thr Gly Val
 65                  70                  75                  80

Val Arg Asp Cys Glu Gly Thr Asp Ala Arg Pro Tyr Cys Lys Val Leu
                 85                  90                  95

Leu Leu His Glu Val His Ala Asn Asp Thr Gly Ser Tyr Val Cys Tyr
            100                 105                 110

Tyr Lys Tyr Ile Lys Ala Arg Ile Glu Gly Thr Thr Ala Ala Ser Ser
        115                 120                 125

Tyr Val Phe Val Arg Asp Phe Glu Gln Pro Phe Ile Asn Lys Pro Asp
    130                 135                 140

Thr Leu Leu Val Asn Arg Lys Asp Ala Met Trp Val Pro Cys Leu Val
145                 150                 155                 160

Ser Ile Pro Gly Leu Asn Val Thr Leu Arg Ser Gln Ser Ser Val Leu
                165                 170                 175

Trp Pro Asp Gly Gln Glu Val Val Trp Asp Asp Arg Arg Gly Met Leu
            180                 185                 190

Val Ser Thr Pro Leu Leu His Asp Ala Leu Tyr Leu Gln Cys Glu Thr
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Thr Trp Gly Asp Gln Asp Phe Leu Ser Asn Pro Phe Leu Val His Ile
    210                 215                 220

Thr Gly Asn Glu Leu Tyr Asp Ile Gln Leu Leu Pro Arg Lys Ser Leu
225                 230                 235                 240

Glu Leu Leu Val Gly Glu Lys Leu Val Leu Asn Cys Thr Val Trp Ala
                245                 250                 255

Glu Phe Asn Ser Gly Val Thr Phe Asp Trp Asp Tyr Pro Gly Lys Gln
            260                 265                 270

Ala Glu Arg Gly Lys Trp Val Pro Glu Arg Arg Ser Gln Gln Thr His
        275                 280                 285

Thr Glu Leu Ser Ser Ile Leu Thr Ile His Asn Val Ser Gln His Asp
    290                 295                 300

Leu Gly Ser Tyr Val Cys Lys Ala Asn Asn Gly Ile Gln Arg Phe Arg
305                 310                 315                 320

Glu Ser Thr Glu Val Ile Val His Glu Asn Pro Phe Ile Ser Val Glu
                325                 330                 335

Trp Leu Lys Gly Pro Ile Leu Glu Ala Thr Ala Gly Asp Glu Leu Val
            340                 345                 350
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Tyr Lys Asp Gly Lys Ala Leu Ser Gly Arg His Ser Pro His Ala Leu
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Val Leu Lys Glu Val Thr Glu Ala Ser Thr Gly Thr Tyr Thr Leu Ala
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Leu Trp Asn Ser Ala Ala Gly Leu Arg Arg Asn Ile Ser Leu Glu Leu
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Val Val Asn Val Pro Pro Gln Ile His Glu Lys Glu Ala Ser Ser Pro
420 425 430

Ser Ile Tyr Ser Arg His Ser Arg Gln Ala Leu Thr Cys Thr Ala Tyr
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Gly Val Pro Leu Pro Leu Ser Ile Gln Trp His Trp Arg Pro Trp Thr
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Pro Cys Lys Met Phe Ala Gln Arg Ser Leu Arg Arg Arg Gln Gln Gln
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Asp Leu Met Pro Gln Cys Arg Asp Trp Arg Ala Val Thr Thr Gln Asp
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Ala Val Asn Pro Ile Glu Ser Leu Asp Thr Trp Thr Glu Phe Val Glu
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Gly Lys Asn Lys Thr Val Ser Lys Leu Val Ile Gln Asn Ala Asn Val
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Ser Ala Met Tyr Lys Cys Val Val Ser Asn Lys Val Gly Gln Asp Glu
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Arg Leu Ile Tyr Phe Tyr Val Thr Thr Ile Pro Asp Gly Phe Thr Ile
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Glu Ser Lys Pro Ser Glu Glu Leu Leu Glu Gly Gln Pro Val Leu Leu
565 570 575

Ser Cys Gln Ala Asp Ser Tyr Lys Tyr Glu His Leu Arg Trp Tyr Arg
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Leu Asn Leu Ser Thr Leu His Asp Ala His Gly Asn Pro Leu Leu Leu
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Asp Cys Lys Asn Val His Leu Phe Ala Thr Pro Leu Ala Ala Ser Leu
610 615 620

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625 630 635 640

Pro Arg Val Ala Pro Glu His Glu Gly His Tyr Val Cys Glu Val Gln
645 650 655

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            660                 665                 670

Gln Ala Leu Glu Ala Pro Arg Leu Thr Gln Asn Leu Thr Asp Leu Leu
        675                 680                 685

Val Asn Val Ser Asp Ser Leu Glu Met Gln Cys Leu Val Ala Gly Ala
    690                 695                 700

His Ala Pro Ser Ile Val Trp Tyr Lys Asp Glu Arg Leu Leu Glu Glu
705                 710                 715                 720

Lys Ser Gly Val Asp Leu Ala Asp Ser Asn Gln Lys Leu Ser Ile Gln
                725                 730                 735

Arg Val Arg Glu Glu Asp Ala Gly Arg Tyr Leu Cys Ser Val Cys Asn
            740                 745                 750

Ala Lys Gly Cys Val Asn Ser Ser Ala Ser Val Ala Val Glu Gly Ser
        755                 760                 765

Glu Asp Lys Gly Ser Met Glu Ile Val Ile Leu Val Gly Thr Gly Val
    770                 775                 780

Ile Ala Val Phe Phe Trp Val Leu Leu Leu Leu Ile Phe Cys Asn Met
785                 790                 795                 800

Arg Arg Pro Ala His Ala Asp Ile Lys Thr Gly Tyr Leu Ser Ile Ile
                805                 810                 815

Met Asp Pro Gly Glu Val Pro Leu Glu Glu Gln Cys Glu Tyr Leu Ser
            820                 825                 830

Tyr Asp Ala Ser Gln Trp Glu Phe Pro Arg Glu Arg Leu His Leu Gly
        835                 840                 845

Arg Val Leu Gly Tyr Gly Ala Phe Gly Lys Val Val Glu Ala Ser Ala
    850                 855                 860

Phe Gly Ile His Lys Gly Ser Ser Cys Asp Thr Val Ala Val Lys Met
865                 870                 875                 880

Leu Lys Glu Gly Ala Thr Ala Ser Glu His Arg Ala Leu Met Ser Glu
                885                 890                 895

Leu Lys Ile Leu Ile His Ile Gly Asn His Leu Asn Val Val Asn Leu
            900                 905                 910

Leu Gly Ala Cys Thr Lys Pro Gln Gly Pro Leu Met Val Ile Val Glu
        915                 920                 925

Phe Cys Lys Tyr Gly Asn Leu Ser Asn Phe Leu Arg Ala Lys Arg Asp
    930                 935                 940

Ala Phe Ser Pro Cys Ala Glu Lys Ser Pro Glu Gln Arg Gly Arg Phe
945                 950                 955                 960
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Arg Ala Met Val Glu Leu Ala Arg Leu Asp Arg Arg Arg Pro Gly Ser
965 970 975

Ser Asp Arg Val Leu Phe Ala Arg Phe Ser Lys Thr Glu Gly Gly Ala
980 985 990

Arg Arg Ala Ser Pro Asp Gln Glu Ala Glu Asp Leu Trp Leu Ser Pro
995 1000 1005

Leu Thr Met Glu Asp Leu Val Cys Tyr Ser Phe Gln Val Ala Arg Gly
1010 1015 1020

Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His Arg Asp Leu Ala Ala
025 1030 1035 1040

Arg Asn Ile Leu Leu Ser Glu Ser Asp Val Val Lys Ile Cys Asp Phe
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Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp Tyr Val Arg Lys Gly
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Ser Ala Arg Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asp
1075 1080 1085

Lys Val Tyr Thr Thr Gln Ser Asp Val Trp Ser Phe Gly Val Leu Leu
1090 1095 1100

Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr Pro Gly Val Gln Ile
105 1110 1115 1120

Asn Glu Glu Phe Cys Gln Arg Leu Arg Asp Gly Thr Arg Met Arg Ala
1125 1130 1135

Pro Glu Leu Ala Thr Pro Ala Ile Arg Arg Ile Met Leu Asn Cys Trp
1140 1145 1150

Ser Gly Asp Pro Lys Ala Arg Pro Ala Phe Ser Glu Leu Val Glu Ile
1155 1160 1165

Leu Gly Asp Leu Leu Gln Gly Arg Gly Leu Gln Glu Glu Glu Glu Val
1170 1175 1180

Cys Met Ala Pro Arg Ser Ser Gln Ser Ser Glu Glu Gly Ser Phe Ser
185 1190 1195 1200

Gln Val Ser Thr Met Ala Leu His Ile Ala Gln Ala Asp Ala Glu Asp
1205 1210 1215

Ser Pro Pro Ser Leu Gln Arg His Ser Leu Ala Ala Arg Tyr Tyr Asn
1220 1225 1230

Trp Val Ser Phe Pro Gly Cys Leu Ala Arg Gly Ala Glu Thr Arg Gly
1235 1240 1245

Ser Ser Arg Met Lys Thr Phe Glu Glu Phe Pro Met Thr Pro Thr Thr
1250 1255 1260

Tyr Lys Gly Ser Val Asp Asn Gln Thr Asp Ser Gly Met Val Leu Ala
265 1270 1275 1280

Ser Glu Glu Phe Glu Gln Ile Glu Ser Arg His Arg Gln Glu Ser Gly
1285 1290 1295

Phe Ser Cys Lys Gly Pro Gly Gln Asn Val Ala Val Thr Arg Ala His
1300 1305 1310

Pro Asp Ser Gln Gly Arg Arg Arg Arg Pro Glu Arg Gly Ala Arg Gly
1315 1320 1325

Gly Gln Val Phe Tyr Asn Ser Glu Tyr Gly Glu Leu Ser Glu Pro Ser
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Glu Glu Asp His Cys Ser Pro Ser Ala Arg Val Thr Phe Phe Thr Asp
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<213> Homo sapiens

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ttttacctga cacccgccgc ctttccccgg cactggctgg gagggcgccc tgcaaagttg 180

ggaacgcgga gccccggacc cgctcccgcc gcctccggct cgcccagggg gggtcgccgg 240

gaggagcccg ggggagaggg accaggaggg gcccgcggcc tcgcaggggc gcccgcgccc 300

ccacccctgc ccccgccagc ggaccggtcc cccacccccg gtccttccac c atg cac 357
Met His
1

ttg ctg ggc ttc ttc tct gtg gcg tgt tct ctg ctc gcc gct gcg ctg 405
Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala Ala Leu
5 10 15

ctc ccg ggt cct cgc gag gcg ccc gcc gcc gcc gcc gcc ttc gag tcc 453
Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Ala Phe Glu Ser
20 25 30

gga ctc gac ctc tcg gac gcg gag ccc gac gcg ggc gag gcc acg gct 501
Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala Thr Ala
35 40 45 50

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tat gca agc aaa gat ctg gag gag cag tta cgg tct gtg tcc agt gta   549
Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser Val
                 55                  60                  65

gat gaa ctc atg act gta ctc tac cca gaa tat tgg aaa atg tac aag   597
Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys
             70                  75                  80

tgt cag cta agg aaa gga ggc tgg caa cat aac aga gaa cag gcc aac   645
Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn
         85                  90                  95

ctc aac tca agg aca gaa gag act ata aaa ttt gct gca gca cat tat   693
Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr
    100                 105                 110

aat aca gag atc ttg aaa agt att gat aat gag tgg aga aag act caa   741
Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln
115                 120                 125                 130

tgc atg cca cgg gag gtg tgt ata gat gtg ggg aag gag ttt gga gtc   789
Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val
                135                 140                 145

gcg aca aac acc ttc ttt aaa cct cca tgt gtg tcc gtc tac aga tgt   837
Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys
            150                 155                 160

ggg ggt tgc tgc aat agt gag ggg ctg cag tgc atg aac acc agc acg   885
Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr
        165                 170                 175

agc tac ctc agc aag acg tta ttt gaa att aca gtg cct ctc tct caa   933
Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln
    180                 185                 190

ggc ccc aaa cca gta aca atc agt ttt gcc aat cac act tcc tgc cga   981
Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg
195                 200                 205                 210

tgc atg tct aaa ctg gat gtt tac aga caa gtt cat tcc att att aga   1029
Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg
                215                 220                 225

cgt tcc ctg cca gca aca cta cca cag tgt cag gca gcg aac aag acc   1077
Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr
            230                 235                 240

tgc ccc acc aat tac atg tgg aat aat cac atc tgc aga tgc ctg gct   1125
Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala
        245                 250                 255

cag gaa gat ttt atg ttt tcc tcg gat gct gga gat gac tca aca gat   1173
Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp
    260                 265                 270
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gga ttc cat gac atc tgt gga cca aac aag gag ctg gat gaa gag acc   1221
Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr
275                 280                 285                 290

tgt cag tgt gtc tgc aga gcg ggg ctt cgg cct gcc agc tgt gga ccc   1269
Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro
                295                 300                 305

cac aaa gaa cta gac aga aac tca tgc cag tgt gtc tgt aaa aac aaa   1317
His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys
            310                 315                 320

ctc ttc ccc agc caa tgt ggg gcc aac cga gaa ttt gat gaa aac aca   1365
Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr
        325                 330                 335

tgc cag tgt gta tgt aaa aga acc tgc ccc aga aat caa ccc cta aat   1413
Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn
    340                 345                 350

cct gga aaa tgt gcc tgt gaa tgt aca gaa agt cca cag aaa tgc ttg   1461
Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu
355                 360                 365                 370

tta aaa gga aag aag ttc cac cac caa aca tgc agc tgt tac aga cgg   1509
Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg
                375                 380                 385

cca tgt acg aac cgc cag aag gct tgt gag cca gga ttt tca tat agt   1557
Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser
            390                 395                 400

gaa gaa gtg tgt cgt tgt gtc cct tca tat tgg aaa aga cca caa atg   1605
Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met
        405                 410                 415

agc taa gattgtactg ttttccagtt catcgatttt ctattatgga aaactgtgtt   1661
Ser
    420

gccacagtag aactgtctgt gaacagagag acccttgtgg gtccatgcta acaaagacaa 1721

aagtctgtct ttcctgaacc atgtggataa ctttacagaa atggactgga gctcatctgc 1781

aaaaggcctc ttgtaaagac tggttttctg ccaatgacca aacagccaag attttcctct 1841

tgtgatttct ttaaaagaat gactatataa tttatttcca ctaaaaatat tgtttctgca 1901

ttcatttta tagcaacaac aattggtaaa actcactgtg atcaatattt ttatatcatg 1961

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<212> PRT
<213> Homo sapiens

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Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Ala Phe
             20                  25                  30

Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala
         35                  40                  45

Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser
     50                  55                  60

Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met
 65                  70                  75                  80

Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln
                 85                  90                  95

Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala
            100                 105                 110

His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys
        115                 120                 125

Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe
    130                 135                 140

Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr
145                 150                 155                 160

Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr
                165                 170                 175

Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu
            180                 185                 190

Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser
        195                 200                 205

Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile
    210                 215                 220

Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn
225                 230                 235                 240

Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys
                245                 250                 255

Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser
            260                 265                 270

Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu
        275                 280                 285

Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys
    290                 295                 300
```

```
Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys
305                 310                 315                 320

Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu
                325                 330                 335

Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro
            340                 345                 350

Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys
        355                 360                 365

Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr
    370                 375                 380

Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser
385                 390                 395                 400

Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro
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Gln Met Ser


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<213> Homo sapiens

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ttagagtgct ttctaatttc aggtagaaga catgtccacc ttctgattat ttttggagaa 180

cattttgatt tttttcatct ctctctcccc acccctaaga ttgtgcaaaa aaagcgtacc 240

ttgcctaatt gaaataattt cattggattt tgatcagaac tgattatttg gttttctgtg 300

tgaagttttg aggtttcaaa ctttccttct ggagaatgcc ttttgaaaca attttctcta 360

gctgcctgat gtcaactgct tagtaatcag tggatattga aatattcaaa atg tac    416
                                                        Met Tyr
                                                          1

aga gag tgg gta gtg gtg aat gtt ttc atg atg ttg tac gtc cag ctg   464
Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val Gln Leu
          5                  10                  15
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gtg cag ggc tcc agt aat gaa cat gga cca gtg aag cga tca tct cag   512
Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser Ser Gln
     20                  25                  30

tcc aca ttg gaa cga tct gaa cag cag atc agg gct gct tct agt ttg   560
Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser Ser Leu
 35                  40                  45                  50

gag gaa cta ctt cga att act cac tct gag gac tgg aag ctg tgg aga   608
Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu Trp Arg
                 55                  60                  65

tgc agg ctg agg ctc aaa agt ttt acc agt atg gac tct cgc tca gca   656
Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg Ser Ala
             70                  75                  80

tcc cat cgg tcc act agg ttt gcg gca act ttc tat gac att gaa aca   704
Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile Glu Thr
         85                  90                  95

cta aaa gtt ata gat gaa gaa tgg caa aga act cag tgc agc cct aga   752
Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser Pro Arg
    100                 105                 110

gaa acg tgc gtg gag gtg gcc agt gag ctg ggg aag agt acc aac aca   800
Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr Asn Thr
115                 120                 125                 130

ttc ttc aag ccc cct tgt gtg aac gtg ttc cga tgt ggt ggc tgt tgc   848
Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly Cys Cys
                135                 140                 145

aat gaa gag agc ctt atc tgt atg aac acc agc acc tcg tac att tcc   896
Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr Ile Ser
            150                 155                 160

aaa cag ctc ttt gag ata tca gtg cct ttg aca tca gta cct gaa tta   944
Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro Glu Leu
        165                 170                 175

gtg cct gtt aaa gtt gcc aat cat aca ggt tgt aag tgc ttg cca aca   992
Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu Pro Thr
    180                 185                 190

gcc ccc cgc cat cca tac tca att atc aga aga tcc atc cag atc cct  1040
Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln Ile Pro
195                 200                 205                 210

gaa gaa gat cgc tgt tcc cat tcc aag aaa ctc tgt cct att gac atg  1088
Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile Asp Met
                215                 220                 225

cta tgg gat agc aac aaa tgt aaa tgt gtt ttg cag gag gaa aat cca  1136
Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu Asn Pro
            230                 235                 240
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ctt gct gga aca gaa gac cac tct cat ctc cag gaa cca gct ctc tgt 1184
Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala Leu Cys
245 250 255

ggg cca cac atg atg ttt gac gaa gat cgt tgc gag tgt gtc tgt aaa 1232
Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val Cys Lys
260 265 270

aca cca tgt ccc aaa gat cta atc cag cac ccc aaa aac tgc agt tgc 1280
Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys Ser Cys
275 280 285 290

ttt gag tgc aaa gaa agt ctg gag acc tgc tgc cag aag cac aag cta 1328
Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His Lys Leu
295 300 305

ttt cac cca gac acc tgc agc tgt gag gac aga tgc ccc ttt cat acc 1376
Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe His Thr
310 315 320

aga cca tgt gca agt ggc aaa aca gca tgt gca aag cat tgc cgc ttt 1424
Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys Arg Phe
325 330 335

cca aag gag aaa agg gct gcc cag ggg ccc cac agc cga aag aat cct 1472
Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys Asn Pro
340 345 350

tga ttcagcgttc caagttcccc atccctgtca tttttaacag catgctgctt 1525

355

tgccaagttg ctgtcactgt ttttttccca ggtgttaaaa aaaaaatcca ttttacacag 1585

caccacagtg aatccagacc aaccttccat tcacaccagc taaggagtcc ctggttcatt 1645

gatggatgtc ttctagctgc agatgcctct gcgcaccaag gaatggagag gaggggaccc 1705

atgtaatcct tttgtttagt tttgtttttg ttttttggtg aatgagaaag gtgtgctggt 1765

catggaatgg caggtgtcat atgactgatt actcagagca gatgaggaaa actgtagtct 1825

ctgagtcctt tgctaatcgc aactcttgtg aattattctg attcttttt atgcagaatt 1885

tgattcgtat gatcagtact gactttctga ttactgtcca gcttatagtc ttccagttta 1945

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<213> Homo sapiens

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            20                  25                  30

Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
        35                  40                  45

Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu
    50                  55                  60

Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg
65                  70                  75                  80

Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile
                85                  90                  95

Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
            100                 105                 110

Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr
        115                 120                 125

Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
    130                 135                 140

Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr
145                 150                 155                 160

Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
                165                 170                 175

Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu
            180                 185                 190

Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
        195                 200                 205

Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile
    210                 215                 220

Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu
225                 230                 235                 240

Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala
                245                 250                 255

Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val
            260                 265                 270

Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys
        275                 280                 285

Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His
    290                 295                 300
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Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe
305 310 315 320

His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys
325 330 335

Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys
340 345 350

Asn Pro

<210> 7
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 7
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<210> 8
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
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oligonucleotide

<400> 8
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<210> 9
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<212> DNA
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<220>
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oligonucleotide

<400> 9
caggacgggg tgacttga 18

<210> 10
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<213> Artificial Sequence

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<223> Description of Artificial Sequence:oligonucleotide

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<210> 11
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<220>
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<210> 16
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<220>
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<210> 18
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<220>
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<400> 18
ggtgcctccc tgtaccctgg g 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06133

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06133

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/68; A61K 38/00, 38/19, 48/00; C07H 21/02, 21/04; C12N 15/11; C12P 19/34; G01N 27/26

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 91.2; 424/85.1; 514/2, 44, 536/23.1; 536/24, 33, 24, 31, 24.3; 204/450

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-10, drawn to methods of screening humans for lymphatic disorders by detecting mutations.
Group II, claim(s) 11-12, drawn to methods of treatment.
Group III, claim(s) 13-17, drawn to oligonucleotides.
Group IV, claim(s)18-21, drawn to vectors and host cells.
Group V, claim(s) 22-25, drawn to methods of identifying compounds.
The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The mutations in the nucleic acid sequence of VEGFR-3 associated with disease, which is considered the technical feature linking all the claims, were well known in the art at the time of filing as taught by Boultwood(1994, Genomics, Vol. 19, pages 425-432). It would have been obvious to detect mutations in the VEGFR-3 to screen for lymphatic disorder, replace the VEGFR-3 using gene therapy, make oligonucleotides with the mutated VEGFR-3, make vectors and host cells with the mutated VEGFR-3 or identify compounds having an effect on cells with mutated VEGFR-3.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06133

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 424/85.1; 514/2, 44, 536/23.1; 536/24, 33, 24, 31, 24.3; 204/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Caplus, biosis, medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THOMPSON et al. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev. Biology. 15 May 1998, Vol. 197, No. 2, pages 248-269, see entire article.	13-25
Y	FERRELL et al. Hereditary lymphedema: evidence for linkage and genetic heterogeneity. Hum. Mol. Genetics. December 1998, Vol. 7, No. 13, pages 2073-2078, see entire article.	1-25
Y	FOURNIER et al. Mutation in tyrosine residue 1337 abrogates ligand-dependent transforming capacity of the FLT4 receptor. Oncogene. 1995, Vol. 11, No. 5, pages 921-931, see entire article.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

24 MAY 1999

Date of mailing of the international search report

22 JUN 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06133

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FOURNIER et al. Role of tyrosine residues and protein interaction domains of SHC adaptor in VEGF receptor 3 signaling. Oncogene. 14 January 1999, Vol. 18, No. 2, pages 507-514, see entire article.	1-25
Y	WITTE et al. Phenotypic and genotypic hetherogeneity in familial Milroy lymphedema. Lymphology. December 1998, Vol. 31, No. 4, pages 145-155, see entire article.	1-10
Y	BOULTWOOD et al. Molecular mapping of uncharacteristically small 5q deletions in two patients with the 5q- syndrome: delineation of the critical region on 5q and identification of a 5q- breakpoint. Genomic. 1994, Vol. 19, No. 3, pages 425-432, see entire article.	1-10

Fa
Form

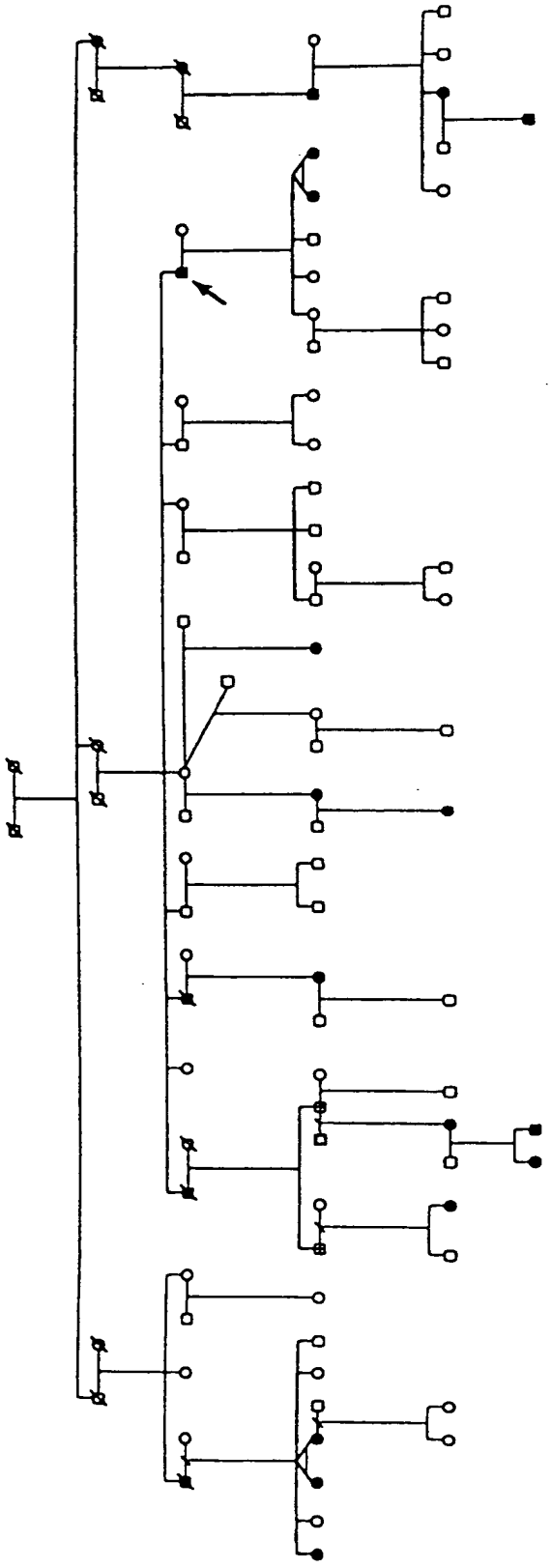


Fig. 1A

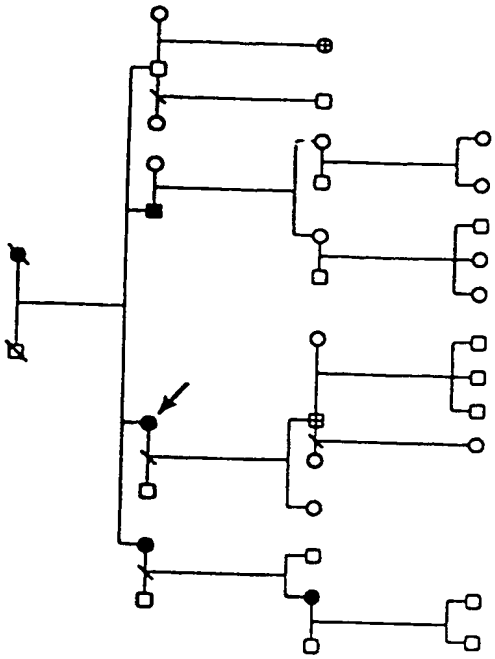


Fig. 1C

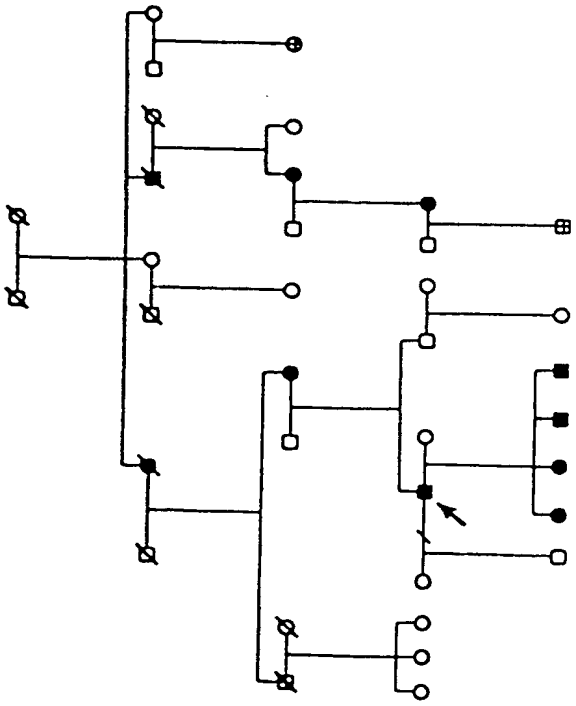
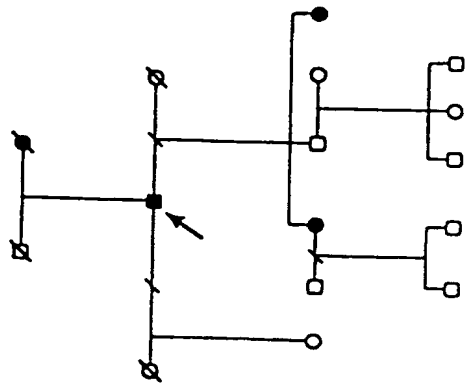


Fig. 1B



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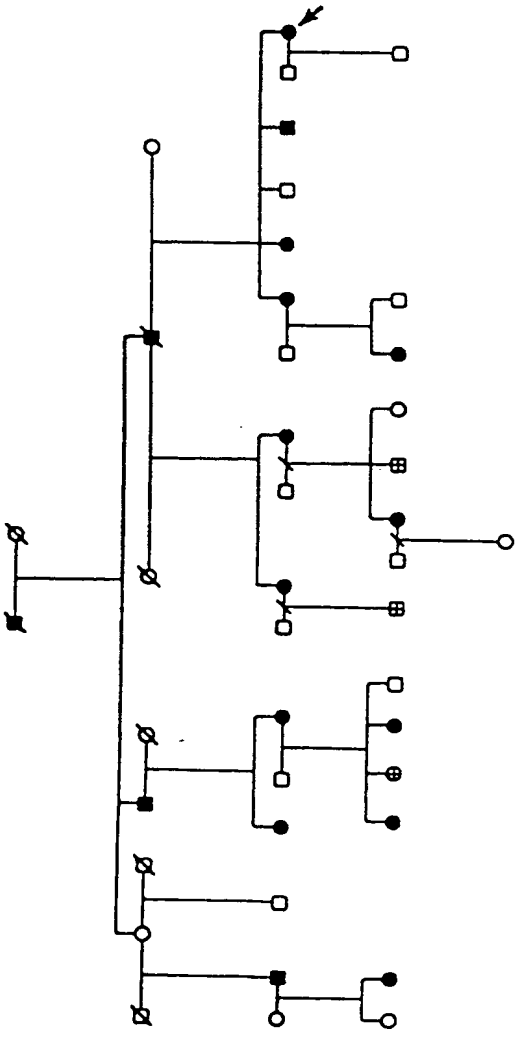

Fig. 1D

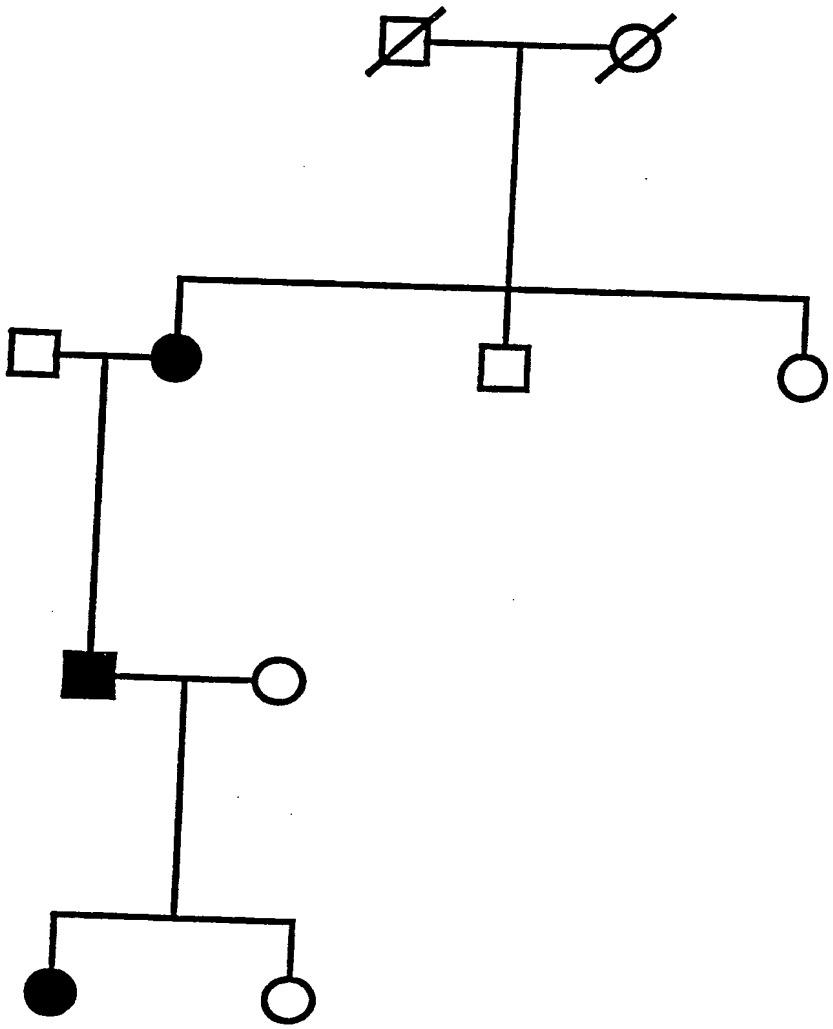

Fig. 1F

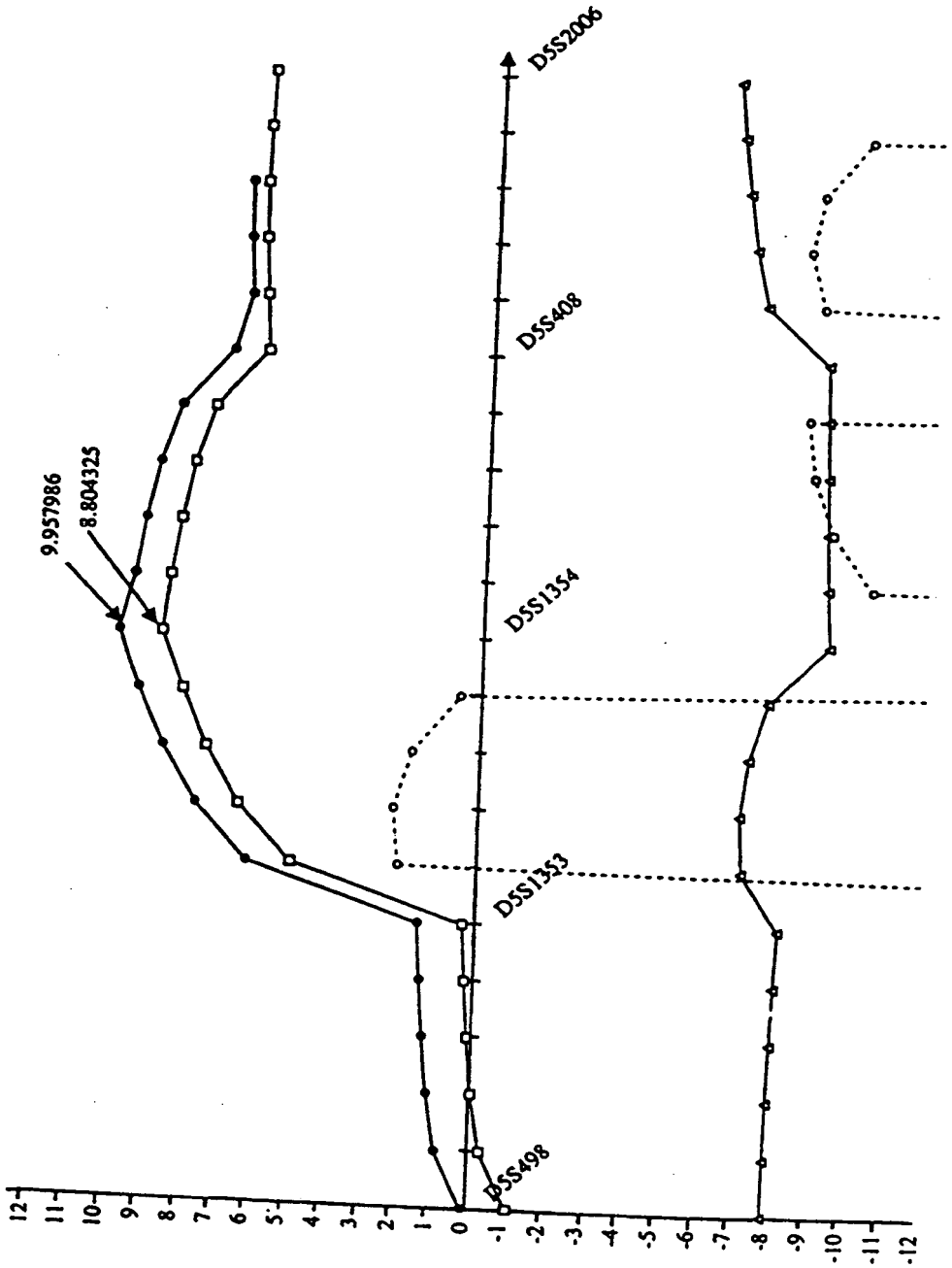
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Fig. 2